**Collagen D-spacing and the Effect of Fat Liquor Addition**

by

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**Abstract**

The physical properties of leather are partly a result of the structure of the leather’s network of type I collagen fibrils. To achieve high strength and a soft, supple feel, penetrating oils (usually polyols) are added to leather during manufacture, and this process is known as fat liquoring. The modification of the collagen structure by fat liquoring (with a lanolin-based fat liquor) is investigated using synchrotron-based small angle X-ray scattering. The axial periodicity, or D-spacing, of the collagen changes as a result of fat liquoring. With no fat liquor, the D-spacing is 60.2 nm; spacing increases by 6% to 63.6 nm at 10% fat liquor. Pure lanolin results in a similar increase in D-spacing. We discuss mechanisms for fibril elongation brought about by fat liquoring. The observations of structural changes taking place within collagen fibrils as a result of fat liquoring provides new insight into the nature of fat liquoring and informs future processing developments.

**Introduction**

Leather is a strong, flexible, complex biomaterial mainly consisting of fibrous type I collagen. Leather is used in a wide variety of manufacturing applications where the physical properties exhibited by the material are important for both strength and aesthetic reasons. The physical attributes of leather are largely dependent on the structure of collagen fibers and the interactions among them. 1,5 Because leather is processed animal skin, the collagen fiber structure of leather is derived from that of living skin and some other tissues. It is also similar to some collagen-based medical scaffolds. 6 Fibrous type I collagen accounts for most of leather’s complex architecture.

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A collagen fibril contains multiple levels of structure. The collagen molecule is characterized by the repeating amino acid sequence (Glycine-X-Y)\textsuperscript{3}. Each polypeptide chain forms an alpha helix with a left handed twist, then three of these left handed helices twist together in a right handed manner to form a triple helix, or tropocollagen. Hydrogen bonds between amino acid side chains and collagen molecule main chains and those mediated through water bridges are the main stabilizing force of the tropocollagen quaternary structure.\textsuperscript{7} Interchain water bridges are intrinsically linked to the hydroxyprolines in the sequence so that high hydroxyproline content will increase the stability of the triple helix.\textsuperscript{8} In fact, water can be regarded as forming a clathrate–like structure around each triple helix and it has a role in maintaining fibril assembly.\textsuperscript{7,9,10} Collagen fibrils are assembled from multiples of five staggered tropocollagens. Gaps between the end of one tropocollagen and the next result in regions with only 4/5 tropocollagen molecules present. This structure is responsible for the banding of collagen that is visible with atomic force microscopy or transmitting electron microscopy and is the origin of the Bragg diffraction peaks. This spacing is known as the D-spacing and can be measured. Within each fibril, the tropocollagen molecules are held together by crosslinks between lysine and allylsine formed as a result of the action of an enzyme lysyl oxidase. The extensive, highly structured hydration shell around the collagen triple helices, along with water bridges between collagen fibrils, have been shown to be critical elements that maintain the macromolecular assemblies of collagen molecules.\textsuperscript{7,11,12} While the secondary structure remains stable if the water molecules become unavailable to support the hydrogen-bonding network, the mechanical properties of collagen are affected.\textsuperscript{13} Hydrophobic residues on the outside of the tropocollagen molecule have also been shown to play an important role in microfibrillar packing by both organizing water structure and through Van der Waals interactions.\textsuperscript{14}

In terms of microstructure, leather comprises two distinct layers: the ‘grain’ and the ‘corium’. These two layers have significantly different structures.\textsuperscript{1,15} Fibril orientation and fibril diameter, particularly in the corium layer, have been shown to be an important factor in the strength of the material.\textsuperscript{14-18} During the process of making leather, synthetic crosslinks are introduced that stabilize the molecular structure of the skin and contribute to its physical properties.\textsuperscript{19-22}

The D-spacing for stronger ovine and bovine leather has been shown to decrease at the interface between the corium and the grain.\textsuperscript{23} While changes in D-spacing have not been shown to correlate with strength in leather\textsuperscript{4,16} and rat tail tendon,\textsuperscript{24} D-spacing does vary with tissue types,\textsuperscript{25} animal species,\textsuperscript{16} age\textsuperscript{26,27} and chemical treatment.\textsuperscript{27,28} It is also possible to observe change in D-spacing when leather is subjected to mechanical stress.\textsuperscript{23} During the processing of skins and hides to leather, efforts are made to optimize the strength, flexibility and feel of leather. Adding penetrating oils, a process known as fat liquoring, improves leather’s texture and flexibility by lubricating the fibers and preventing adhesion between them.\textsuperscript{29} However, little is known about how the addition of fat liquor affects the structure of the collagen fibrils themselves.

Here we report a study of the addition of fat liquor to leather in which we attempt to understand how penetrating oil changes the nanostructure of leather. With this understanding, it may be possible to manipulate the processing to produce leather of higher strength and better feel.

**Experimental**

Ovine pelts were obtained from 5-month-old, early season lambs of breeds with “black faces”, which may include Suffolk, South Suffolk, and Dorset Down. Conventional beamhouse and tanning processes were used to generate leather. The pelts were depilated using a caustic treatment comprising sodium sulfide and calcium hydroxide. The residual keratinaceous material was then removed in a solution of sodium sulfide ranging in concentration from 0.8% to 2.4% for 8–16 h at temperatures ranging from 16°C to 24°C. The pelts were then washed and treated with a proteolytic enzyme, either a bacterial enzyme (Tanzyme, Tryptec Biochemicals, Ltd) or a pancreatic enzyme (Rohapon ANZ, Shamrock, Ltd.), at concentrations ranging from 0.025% to 0.1%, followed by pickling in a 2% sulfuric acid and 10% sodium chloride solution. The pickled pelts were then pretanned using oxazolidine, degreased with an aqueous surfactant, and then tanned using chromium sulfate. The resulting “wet blue” was then retanned using a mimosa vegetable extract.

Fat liquoring was carried out using Lipsol EHF (Schill + Seilacher). This product contains a mixture of lanolin, bisulfited fish oil and 2-methyl-2,4-pentanediol. Lanolin, or wool wax, consists primarily of long chain waxy esters and some hydrolysis and oxidation products of these esters. The fat liquor was added at 0–10% by weight of wet leather prior to drying and mechanical softening. One sample was prepared with just the principal component of the fat liquor, lanolin (Sigma), at a concentration of 8% by weight of wet leather.

Samples for synchrotron-based small angle X-ray scattering (SAXS) analysis were prepared by cutting strips of leather 1 × 30 mm from the official sampling position (OSP)\textsuperscript{30} from pelts of leather processed with 0, 2, 4, 6, 8, and 10% Lipsol EHF. Diffraction patterns were recorded on the Australian Synchrotron SANS/WAXS beamline using a high-intensity undulator source. Each sample was mounted without tension in the X-ray beam to obtain scattering patterns from an edge-on direction. Measurements were made every 0.25 mm through the cross section from the grain to the corium. Energy resolution of 10\textsuperscript{-4} was obtained from a cryo-cooled Si (111) double-crystal monochromator, and the beam size [full width
at half maximum (fwhm) focused at the sample] was \( 250 \times 80 \) μm, with a total photon flux of about \( 2 \times 10^{12} \) photons s\(^{-1}\). All diffraction patterns were recorded with an X-ray energy of 11 keV using a Pilatus 1 M detector with an active area of \( 170 \times 170 \) mm and a sample–detector distance of 3371 mm. Exposure time for diffraction patterns was 1 s, and data processing was carried out using the SAXS15ID software.\(^{31}\)

The D-spacing of collagen was determined for each spectrum from Bragg’s Law by taking the central position of several collagen peaks, dividing these by the peak order (usually from \( n = 5 \) to \( n = 10 \)) and averaging the resulting values.

Fibril diameters were calculated from the SAXS data using the Irena software package\(^{32}\) running within Igor Pro. The data were fitted at the wave vector \( Q \) in the range of \( 0.01 – 0.04 \) Å\(^{-1}\) and at an azimuthal angle which was 90° to the long axis of most of the collagen fibrils. This angle was selected by determining the average orientation of the collagen fibrils from the azimuthal angle for the maximum intensity of the d-spacing diffraction peaks. The “cylinderAR” shape model with an arbitrary aspect ratio of 30 was used for all fitting. We did not attempt to individually optimize this aspect ratio and the unbranched length of collagen fibrils may in practice have a length that exceeds an aspect ratio of 30.

**RESULTS**

The SAXS patterns obtained for the different levels of fat liquor clearly show diffraction rings due to the axial periodicity of collagen (Figure 1a). Orientation of the collagen fibrils can be seen as the varying intensity of each of these rings around the azimuthal angle and the alignment at right angles to this of the central scattering region. From the integrated intensity of the whole scattering pattern (Figure 1b) the position of the diffraction peaks can be measured and from these the D-spacing is determined.

The addition of fat liquor resulted in an increase in D-spacing of the collagen from 60.2 (σ = 0.47) nm for samples with no fat liquor to 63.6 (σ = 0.43) nm for samples with 10% fat liquor (Figure 2). This is an increase of 3.4 nm or 5.6%. The change in D-spacing of the corium and grain layers closely mimicked each other despite structural differences in these layers.\(^{33}\) We find a strong correlation between D-spacing and the percentage of fat liquor added, with a linear fitted slope of 0.34 nm/% and a \( r^2 \) value of 0.93 (\( P = 0.0018 \) at an alpha of 0.05) (Figure 2).

The one sample prepared with 8% lanolin rather than fat liquor had a D-spacing of 63.1 (σ = 0.39) nm, which falls on the regression line in Fig. 2. This suggests the change in D-spacing may be primarily due to the lanolin content of the fat liquor.

An average fibril diameter of 56.8 (\( n = 106, \sigma = 1.3 \)) nm was determined for the leather showing that there was no statistically significant change in the fibril diameter as a result of fat liquor addition.

**DISCUSSION**

We found that the change in collagen D-spacing is proportional to the amount of fat liquor added, with a large change being observed when the greatest amount of fat liquor was added. As pure lanolin had a similar effect, it is not unreasonable to assume that the lanolin component of the fat liquor is causing this change. This increase in D-spacing serves to increase the length (and therefore perhaps the volume) of the collagen fibrils in leather. The equivalent extension in D-spacing by tension applied to leather requires a stress of approximately 3.1 N/mm\(^2\) for strong ovine leather and 0.4 N/mm\(^2\) for weak ovine leather.\(^{23}\)
Fat liquor is considered in the industry to assist in the mechanical properties of leather by “lubricating” the fibril structure, enabling the fibers to slide over one another. This work clearly shows that the fat liquor used in this experiment causes a change in the D-spacing, a fundamental property of fibril structure.

We consider two possible mechanisms for the change in D-spacing. One proposition is that the D-spacing increase is caused by an increase in the twist of the tropocollagen helix, which would result in a longer tropocollagen and therefore a longer D-spacing in the fibril. However, if an increase in tropocollagen occurred by this mechanism, we would expect to also see a significant change in the fibril diameter. The diameter was not observed to change and therefore this hypothesis is not supported.

The other option we considered is that the observed change is due to an increase in the length of the gaps between two tropocollagen molecules within the fibrils. Collagen fibrils form in a process that controls the registration between adjacent tropocollagen molecules, known as the D-spacing. Inspection of type I tropocollagen maps and interactomes shows that the two regions of the molecule important in fibrillogenesis (residues ~1016-1040, and 776-800) are rich in hydrophobic amino acids and prolines. Once the fibrillogenesis regions are in register, the fibril structure is stabilized by intermolecular hydrogen bonds, often mediated by water, and covalent crosslinks at the N and C termini of the molecule.

An increase in the D-spacing is indicative of an increase in the axial distance between fibrils and could result in an overall lengthening of the fibril. It could also change the interactions between tropocollagens, whether the interactions are covalent or non-covalent and whether they are mediated through bridging molecules such as water. The fact that it is a direct result of fat liquoring implies that the covalent crosslinks formed between tropocollagen molecules in the mature fibril, are either nonexistent, are broken during processing of the skin, or that they are flexible enough to allow movement of the tropocollagens relative to one another within the fibril.

Axial periodicity in collagen I is thought to be stabilized by inter-tropocollagen hydrophobic and n-CH2 interactions between the C-terminal region of one tropocollagen and a specific region of a second tropocollagen, driven by the entropic gain from the release of ordered water molecules. Lanolin is a long chain hydrocarbon ester, made up from a long chain aliphatic lanolin alcohol and fatty acid. Its hydrophobic structure will therefore be relatively rigid allowing it to insert into the hydrophobic regions at the ends of the gap regions between tropocollagen molecules. The relative non-specific nature of hydrophobic interactions is tolerant of movement, and the lanolin will act like lubricant allowing the tropocollagens to move relative to one another, altering the D-spacing.

It is outside of the scope of this work to elucidate the molecular details of this arrangement. While atomic force microscopy could be used to compare the ratio of the gap and overlap regions among leathers with different levels of fat liquor, obtaining statistically robust sample data could be problematic. It has been shown that there is a range of D-spacings in any one sample, with different fibril bundles within one piece of tissue having different average D-spacings. SAXS measurements sample a volume of $80 \times 150 \times 1000 \, \mu m$, which might be expected to contain around $3 \times 10^{10}$ collagen D-spacing units (assuming collagen occupies around 50% of the volume). AFM, however, scans only small areas of a sample at one time, making it difficult to get a representative average D-spacing. Nevertheless, this would be a worthwhile follow-up analysis and could reveal one mechanism by which fat liquor achieves fibril elongation.

**Conclusions**

In summary, we have investigated the structural changes of collagen within leather upon addition of varying amounts of fat liquor. We have shown that as we increased the amount of fat liquor, the D-spacing of the collagen fibrils increased, and that this appears to be due to the lanolin component of the fat liquor. Alternative mechanisms for this increase in D-spacing have been discussed, including an increase in the gap region or an increase in the length of the tropocollagen triple helix. We have shown that fat liquor does more than lubricate fibers in leather in that it alters the structure of collagen fibrils.

While the focus of this work has been the improved properties of leather, due to the application of lanolin, the changes observed in collagen structure may also occur in raw skin. Mixtures of oils and other chemicals have long been applied to human skin to enhance its appearance and are known as moisturizers. It may be that some of the components of moisturizers increase the D-spacing of collagen in skin, expanding the collagen structure and reducing wrinkles. We are currently investigating the effect of a range of other organic additives on collagen structure as well as the effects of fat liquoring on the nanostructural response of leather when under strain.

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