STRONG SKIN, IS NOT ALWAYS THICK: COMPARATIVE STRUCTURAL AND MOLECULAR ANALYSIS OF DEER SKIN AND COW HIDE

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Abstract. A comprehensive analysis of the molecular and structural components of deer skin and cow hide was undertaken. These skins are known to be strong. However, they derive their strength from different combinations of molecular and structural properties. Firstly, the physical properties of deer skin and cow hide including tensile strength, tear strength, and denaturation temperature were measured. Secondly, the structure of the collagen fibrils and glycosaminoglycans was investigated using transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS). Finally, the chemical composition of deer skin and cow hide, such as amino acids, crosslinks and glycosaminoglycans, were analyzed. Our results showed that the physical properties of deer skin and cow hide are derived from different combinations of several chemical components, resulting in a different architecture. It was found that the large and "wavy" collagen fibers in deer skin are made up of collagen fibrils with small diameters. Additionally, deer skin fibrils appeared to be linked by regular arrays of filaments of large glycosaminoglycans that are distributed uniformly. Deer skin contained a higher proportion of trivalent collagen crosslinks. In contrast, the collagen fibrils in cow hide were larger, contained a diverse glycosaminoglycan distribution and a higher proportion of tetravalent collagen crosslinks, resulting in straight collagen fibers. This study suggests that although deer skin and cow hide are both strong, they have different structural and molecular features.

1 Introduction

Every year billions of animals are slaughtered for meat, producing millions of tons of hides and skins. These are converted to leather which is considered the most significant economic co-product of the meat industry. New Zealand hides and skins contribute to the world's leather industry by providing raw skins and hides for the tanning industry. Leather is used for many manufactured products because of its physical and aesthetic properties [1]. One of these properties is strength, which is critical for many leather products, especially footwear. Leather is manufactured by stabilizing the fibrous collagen networks of animal skins using chemical reagents, a process that is colloquially known as tanning [1]. The origin of the skins and the processing methods used in tanning play a crucial role in determining the properties of the final leather product. Different animal skins and hides, with different physical characteristics, are used to make leather. Strong leather is used for footwear and upholstery while weaker softer leather is used for clothing. Skins from cow, goat, and deer produce strong leather, while sheep skins from dual-purpose sheep produce relatively weak leather [1]. For this study, deer skin and cow hide were chosen, because they are commonly used in the New Zealand tanning industry. Deer skin is thin while cow hide is thick; however, both produce strong leather.

Skin has a complex structure composed mainly of collagen and elastin fibers that associate with proteoglycans [2]. Collagen is the major structural protein and the main component of skin. Collagen type I is the major collagen, making up 70% of dry skin weight, followed by collagen III which makes up 10% [3] . Structurally, skin is composed of three well-defined layers the epidermis, dermis, and flesh layer (hypodermis) [2]. The dermis layer accounts for 90 % of the weight of skin

and is named as the grain and corium layers in the leather industry [1]. The grain layer has a fine and loose collagen fibrous structure with a larger proportion of collagen III and is responsible for the distinctive appearance of leather [1]. The corium layer contains a thicker and more compact collagen fiber network running parallel to the skin surface that imparts strength to the skin [1].
Increasing demand for information about the quality of leather produced from different animal skins and hides has required a better understanding of the molecular differences of skins and hides. To address the structural and molecular factors that affect skin and hide properties, the amino-acid and cross-link composition and structure of deer skin (thin and strong) and cow hide (thick and strong) were analysed using analytical methods and confocal, transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS) respectively. The results from this study will help inform the leather industry to enhance the physical properties of skins and hides by suggesting modifications to existing leather processes.

2 Experimental Procedure

2. 1 Chemicals and materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except for the following: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) which was purchased from SYNCHEM (Altenburg, Germany); the Blyscan glycosaminoglycan assay kit purchased from Biocolor Ltd. (Northern Ireland); mass spectrometry grade water, acetonitrile, methanol and formic acid (>99%) purchased from Fisher Chemical (Fair Lawn, NJ, USA); hydrochloric acid and acetic acid purchased from Panreac (Barcelona, Spain); n-butanol (97%) purchased from Ajax Finechem, Univar (TarenPoint, NSW, Australia); dihydroxylysinonorleuince (DHLNL) purchased from Santa Cruz Biotechnology (Delaware Ave, CA, USA). HLNL, HHL, and HHMD were isolated and purified in our laboratory. Potassium permanganate, sodium tungstate, toluidine blue, and uranyl acetate were purchased from BDH (Poole, England); phosphomolybdic acid from Hopkins and Williams (Essex, England); Sirius red from F3B Spectrum (CA, USA); picric acid from VWR Chemicals (PA, USA); xylene from Labscan (Thailand); glutaraldehyde from Merck (NJ, USA); cuprolinic blue from Polysciences (PA, USA); deuterium oxide (99.9 atom%) (Cambridge Isotope Laboratories, catalog number: DLM-4-100); heparan sulfate (Celsus Laboratories, PN HO3105, Batch HS10697) Tert-butanol (t-BuOH, ACS reagent) (Sigma-Aldrich, catalog number: 360538).

2.2 Skin and hide samples

Deer skin and cow hide were obtained through the New Zealand Leather and Shoe Association Inc. (LASRA®). In summary, the skins and hides were removed from the carcass and immediately chilled to under 8 oC by washing with chilled water through a rotary screen. The skins and hides were then transported to LASRA before the hair was removed from each piece of skin/ hide (1.0 cm × 3.0 cm), cut from the official sampling position (OSP). The samples were then cut parallel to the animal backbone to obtain three technical replicates for each orientation. The thickness of each skin sample was measured using an instrument developed by Wodzicka (1958), which has an accuracy of 0.01 mm [4].

2.3 Tear and tensile strength

Both tear and tensile strength were carried out on fresh skins using a Texture Analyzer (Stable Micro Systems, model TA.XT Plus, Surrey, UK) and according to the international standards ISO 3377-2:2002 and ISO 3376:2011 respectively.

2.4 Small angle x-ray scattering (SAXS)

Scattering patterns of the fresh samples were recorded using the Australian Synchrotron SAXS/WAXS beamline [5-9]. The X-ray beam size was 50 × 50 µm, the wavelength 1.0332 Å, and the instrument calibrated using a silver behenate standard. Diffraction patterns were recorded using a Pilatus 1 M detector with an exposure time of 2 s and a sample to detector distance of 3342 mm, giving a q-range of 0.002 to 0.25 $Å^{-1}$. Data analysis was performed using in-house software to extract the scattering intensity from the raw data image [7].

2.5 Microscopy

Polariser light microscopy (PLM), laser scanning confocal microscopy (LSCM) and transmission electron microscopy (TEM) were carried out on fresh samples [7]. Sample sections were examined with a light microscope and photographed using a Nikon Eclipse E600WPOL polarising light microscope (Nikon Instruments, Melville, New York, USA) at magnifications from 1X to 10X to select the sections to be examined by laser scanning confocal microscopy. For LSCM, samples Skin samples were cut into small pieces (20 mm × 20 mm) then stained with picrosirius red [7, 10, 11]. Sample sections were sliced into a thickness of 40 µm then examined using a Leica SP5 DM6000B confocal microscope (Leica Microsystems Ltd, Knowlhill, Milton Keynes, UK) at different magnifications by capturing one image every 0.05-0.3 µm to generate 3D images [11]. Skin samples were cut into thin slices (1.0 mm) then fixed in a solution containing 2.5 % (v/v) glutaraldehyde and treated with 0.5 % sodium tungstate in acetate buffer for 1 hour then overnight in 0.5 % sodium tungstate in 30 % ethanol before being embedded with resin [12]. The embedded Samples were then examined with a FEI Technai G2 Spirit BioTWIN Transmission Electron Microscope (Czech Republic).

2.6 Lipid, carbohydrate, glycosaminoglycan, amino acid, and crosslink analysis

Lipids, including phospholipids, triglycerides, diglycerides, monoglycerides, sterols, sterol esters, free fatty acids, and others, were extracted from skin samples using the Folch method and analyzed on thin chromatography using three different stains [12, 13]. Glycosaminoglycans in skin samples were determined using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Ltd., Carrickfergus, Co Antrim, United Kingdom). Measurement of carbohydrate was carried out using the phenol-sulfuric acid method [14]. Amino acids and crosslinks in skin and hide were analyzed using our previously published method [15, 16].

3 Results and discussion

3.1 Thickness, tear and tensile strength of deer skin and cow hide

Thickness, tear and tensile strength of deer skin and cow hide are listed in table 1. There is a significant difference in the thickness of deer skin and cow hide with a thickness of 1.6 mm and 4.6 mm respectively (table 1). On the other hand, the tensile strength of deer skin and cow hide is statistically the same (P = 0.05). However, tear strength of deer skin of 130.1 N/mm is much lower than that of cow hide of 228.9 N/mm. We have previously reported that deer skin and cow hide showed typical stress-strain curves with the toe, heel and linear regions [7, 12]. However, we showed that deer skin had the longest toe region with the shallowest slope and cow hide had the shortest and steepest toe region.

Table 1. Thickness, tear and tensile strength of deer skin and cow hide.

Skin	Thickness (mm)	Tensile strength (N/mm^2)	Tear strength (N/mm)
Deer	1.6	28.3	130.1
Cow	4.6	29.2	228.9

3.2 SAXS of deer skin and cow hide

We have previously optimized the experimental conditions to obtain an x-ray scattering pattern which represents the true fibril diameters, d-banding, and orientations in the skin and hide [5-9, 12, 17]. The sum of the diffraction rings from 2 to 6 was chosen to determine the collagen fibril diameters and d-periodicity [7]. The fibril diameter of deer skin is smaller than that of cow hide; however, both d-banding of deer skin and cow hide is the same (Table 2).

Table 2. The fibril diameter and collagen d-banding of deer skin and cow hide as determined by SAXS experiment.

	Deer skin	Cow hide
Fibril diameters (Å)	1308 (± 2.0 %)	1438 (± 5.5 %)
d-banding (Å)	650 (0.02 %)	650 (0.16 %)

3.3 Polariser light microscopy (PLM), laser scanning confocal microscopy (LSCM) and transmission electron microscopy (TEM) of deer skin and cow hide

The polariser light microscope images show that the diameters of the collagen fibers in deer skin are much smaller than those seen in cow hide (Figure 1 & 2).

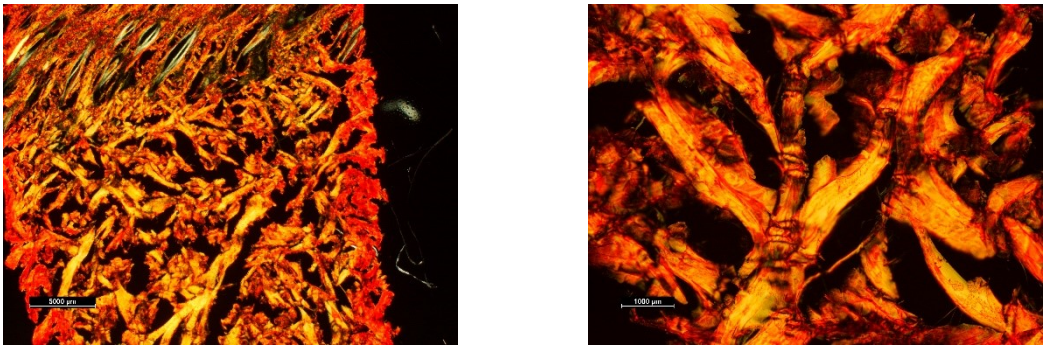

Figure 1. Polariser Light microscopy of the cow hide (scale bar 5000 µm and 1000 µm).

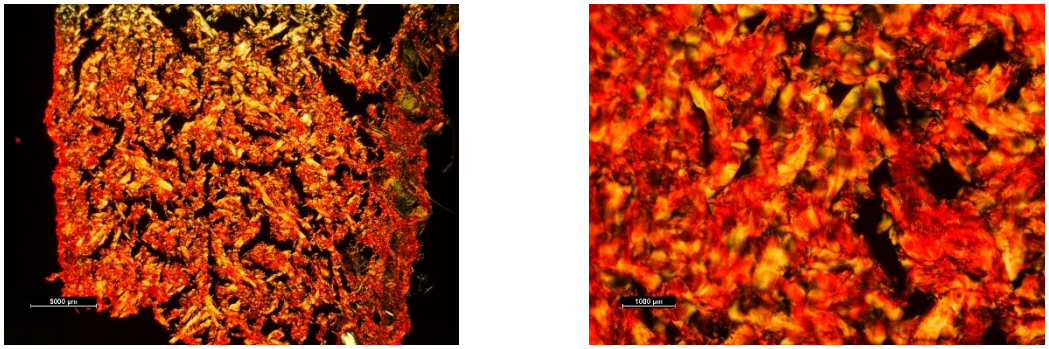

Figure 2. Polariser Light microscopy of the deer skin (scale bar 5000 µm and 1000 µm).

The images of deer skin and cow hide under the laser scanning confocal microscope show that the structural appearance of the grain layer including the organization and size of collagen fibers for both deer skin and cow hide is similar (Figure 3A & 3C). However, a significant difference is seen in the apparent collagen fiber structure in the corium layer (Figure 3B & 3D). It appears that deer skin contains collagen fibers that are smaller and wavier than those seen in cow hide.

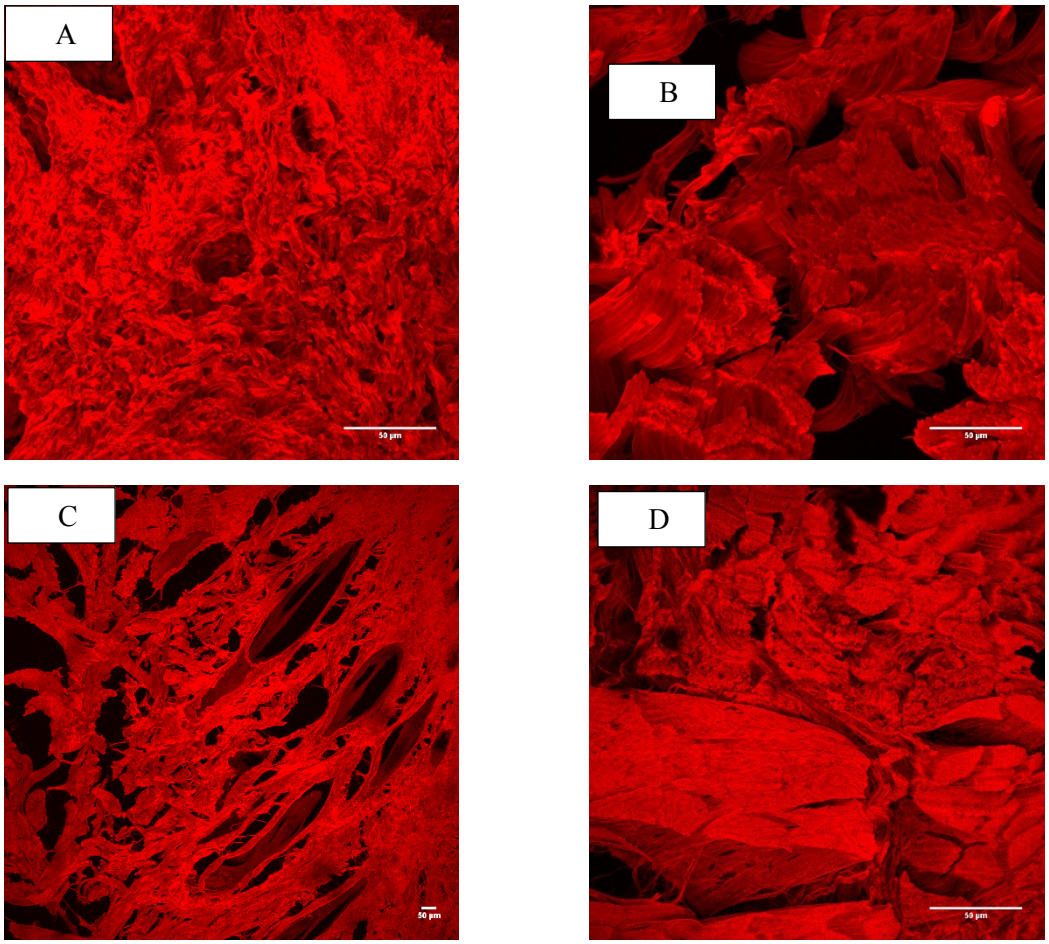

Figure 3. Laser scanning confocal microscopy (LSCM) of grain and corium layers of deer skin and cow hide. A) grain layer of deer skin, B) corium layer of deer skin, C) grain layer of cow hide and D) corium layer of cow hide.

The TEM images of deer skin and cow hides are shown in figure 4. The apparent size of the collagen fibrils of deer skin is smaller than those of cow hide. Also, the fibril bundle of collagen in deer skin appears to have fewer and smaller fibrils compared to cow hide.

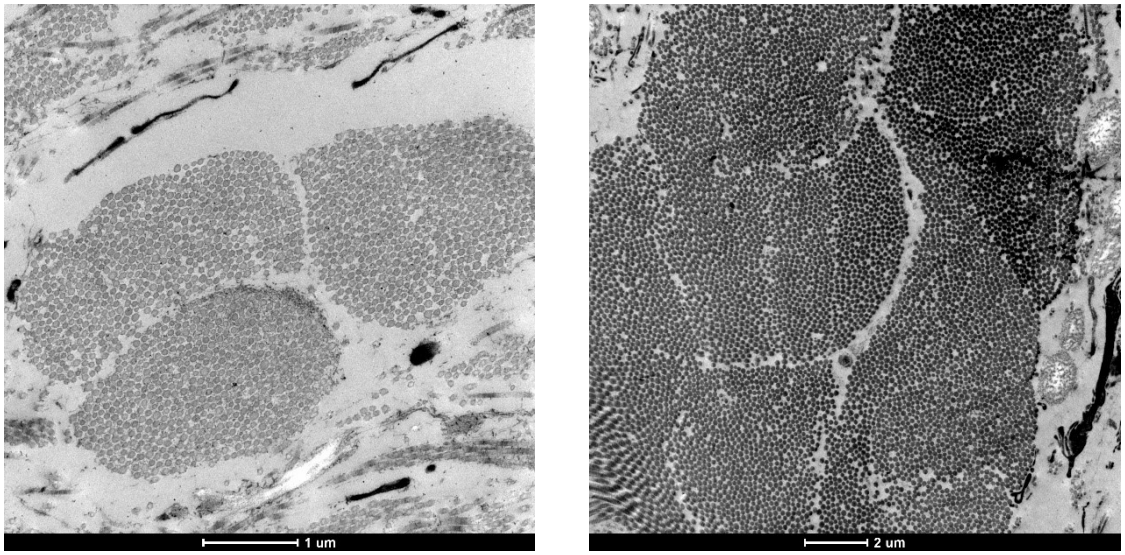

Figure 4. TEM of deer skin (Left) and cow hide (Right).

3.4 Lipid, carbohydrate, glycosaminoglycan, amino acid, and crosslink analysis

Table 3 summarises the molecular composition of deer skin and cow hide. The total protein in deer skin (88.7 %) is lower than that measured in cow hide (92.6 %), and this is associated with the lower collagen content in deer skin of (61.9 %) compared to cow hide (70.9 %). Similar to protein, the grease content in deer skin is lower than that of cow hide with 3.7 % and 5.8 % respectively. The collagen crosslinks are the same in both deer skin and cow hide which is in agreement with previous reports [7, 17]. The ratio between mature (HHL and HHMD) to immature crosslinks (HLNL and DHLNL) shows that deer skin contains a much higher ratio than cow hide

Table 3. Lipid, carbohydrate, glycosaminoglycan, amino acid; and crosslink analysis of deer skin and cow hide.

	Deer	Cow
Glycosaminoglycans % (mg/mg dry skin)	0.47 (±0.04)	0.48 (±0.05)
Total protein % in dry skin	88.7 (±8.5)	92.6 (±9.2)
Collagen % in dry skin	61.9 (±2.19)	70.9 (±1.57)
(mature crosslinks)/(immature crosslinks)	30.07	12.44
Grease content %	3.7 (7.0 %)	5.8 (5.5 %)

The analysis of the lipid profile of deer skin and cow hide using the thin layer chromatography (TLC) method shows differences (Figure 4). Deer skin appears to have a higher content of phospholipids, sterols and sterol esters than cow hide. Interestingly, deer skin does not show any spot for triglycerides, unlike cow hide which shows a clear spot (Figure 5).

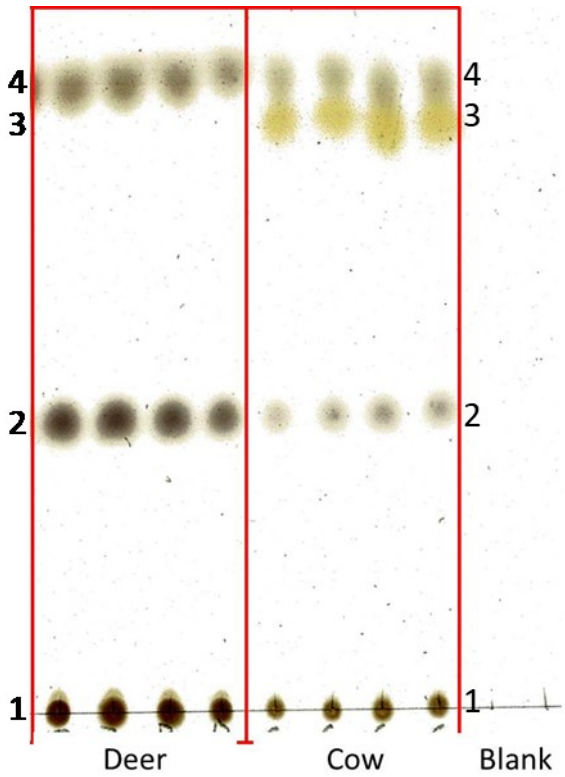

Figure 5. Cellulose TLC of total grease extracts of deer skin and cow hide. Spots 1, 2 and 3 indicate the increased application volumes of total lipid. (1) Phospholipids, (2) Sterols, (3) Triglycerides, (4) Sterol esters. The TLC plate was developed using chloroform then the plate was charred with 10 % H_2SO_4. Spots 4 and 10 were pink before turning black. Blank is the extraction solvents without samples.

4 Conclusion

The molecular composition and collagen structure of deer skin and cow hide were measured including amino acids, collagen crosslinks, glycosaminoglycans and grease content and profile. The collagen structure was then studied using polariser light microscopy (PLM), laser scanning confocal microscopy (LSCM) and transmission electron microscopy (TEM). There was a relationship between the protein, grease and collagen content in deer skin and cow hide and their strength. The amount and profile of grease in deer skin and cow hide was different, where deer skin did not show any presence of triglycerides. Deer skin had a higher ratio of mature to immature crosslinks than cow hide. LSCM showed that collagen fibres in deer skin are smaller and wavier than those seen in cow hide. SAXS data of deer skin and cow hide indicated that both have the same d-banding however deer skin had smaller collagen fibrils while cow hide contained larger collagen fibrils. This study shows that the analysis of the molecular composition and collagen structure of the strong deer skin and strong cow hide helps us to gain a better understanding of the factors that affect physical properties, particularly strength.

5 Acknowledgement

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