Dried Collagenous Biomaterial - Decorin Content and Near Infrared Spectroscopy Analysis

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Abstract:

During the traditional processing of hides to leather, the efficient removal of proteoglycans, such as decorin, is generally acceptable and beneficial for leather quality, especially for softness and flexibility. A patented waterless or acetone dehydration method that can generate a product similar to leather called Dried Collagenous Biomaterial (known as BCD) was developed but the decorin removal efficiency is not known.

The Alcian Blue colorimetric technique was used to assay the sulfated glycosaminoglycan (sGAG) component of decorin. The corresponding residual decorin content was correlated to the mechanical properties of the BCD samples and was comparable to the control leather made traditionally. The waterless dehydration and instantaneous chrome tanning process is a good eco-friendly alternative to transforming hides to leather because no additional effects of acetone were observed after examination using NIR spectroscopy and additional chemometric analysis.

Keywords: proteoglycan; decorin; sulfated glycosaminoglycan (sGAG); Alcian blue; near infrared; principal component analysis (PCA); leather quality

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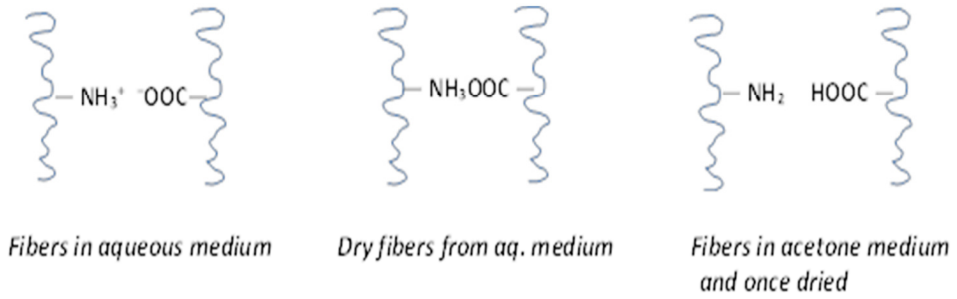
1. Introduction

The current research project deals with the necessity of reducing the burden that traditional tanning of hides to leather impose on the environment by implementing a waterless tanning or dehydration method that can generate a product called Dried Collagenous Biomaterial (known as BCD). The Spanish collaborators conducted the previous studies and prepared the BCD samples at the Association of leather and Related Products Research *Asociación de Investigación de la Industria de Curtidos y Anexas (AIICA*) facility in Igualada, Spain. A patented dehydration method (international patent number PCT / IB2009 / 055733; December, 2009) based on acetone drying and an instantaneous chrome tanning process was utilized (Castell *et al* 2009a; Castell *et al* 2009b). The physical properties of BCD are comparable to leather because it is stabilized, non-putrescible, opaque and flexible. However, BCD is not "tanned", it can be further stabilized by the "instantaneous tanning process" with chrome salts or vegetable extracts. The properties of BCD allow for very fast tanning reactions by immersion of the BCD pelts, therefore, wet blue or vegetable leather can also be obtained from BCD (Castell *et al* 2009a; Castell *et al* 2009b).

Figure 1. Reactive group charges in different medium



To obtain a spongy, flexible and opaque material that has strong solvent absorption capacity, the fibers must remain separated during the drying process by changing the medium polarity from that of water to that of a less polar organic solvent such as acetone as depicted in Figure 1 above (Castell *et al* 2009a; 2009b and 2009c).

Decorin is a small extracellular matrix proteoglycan involved in several fundamental biological functions, including "decorating" or as a glue in the organization of collagen fibrils in animal skin (Scott et al 2004). A decorin molecule consists of a core-protein and a carbohydrate side chain, glycosaminoglycan, or dermatan sulfate (sGAG). Depending on tissue and species, the core protein has a molecular weight of about 40 kDa and the dermatan side chain has a molecular

weight that varies between 20-50 kDa (Mozersky et al 2003; Ramamurthy et al 1996). In nature, the Leucine-rich repeats (LRR) segments of the core decorin protein adopt a stacked β-sheet α-helix hairpin structure resulting in an overall horseshoe shape structure that tends to dimerize as its stable configuration (Scott et al 2004; Ramamurthy et al 1996). The effects of decorin removal on the ultimate properties of the leather made from the differently treated hides were previously determined (Aldema-Ramos *et al* 2008, and 2010). USDA-ARS scientists previously established that as the decorin content is diminished, the leather product becomes softer, more stretchable, and tougher compared to the control leather tanned traditionally (Aldema-Ramos *et al* 2008, and 2010). AIICA and USDA-ARS researchers are particularly interested in the relationship of the available residual decorin content to the mechanical properties of leather product obtained from the BCD materials.

2. Materials and Methods

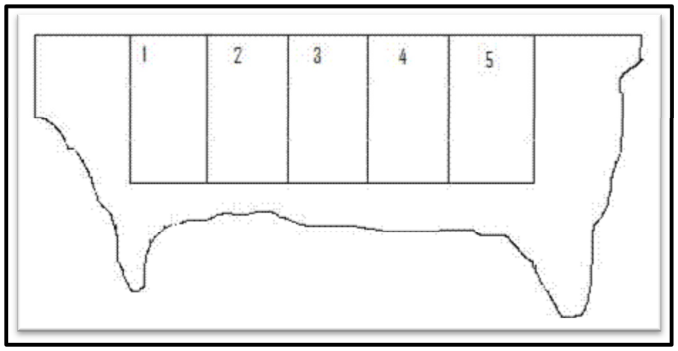
Materials

The SGAG assay Kit was obtained from Kamiya Biochemicals Inc. (Seattle, WA) (Assay Kit manual 2006). Hydroxylamine hydrochloride, Sodium hydroxide and Guanidine hydrochloride (GuHCl): #G4505: were from Sigma-Aldrich (St. Louis, MO, USA). Boron TS and Rohapon 6000 were from TFL USA/Canada (Greensboro, NC, USA). Proxel was from Chemtan Co. (Exeter, NH, USA). Protease inhibitor cocktail for mammalian tissues (#P-8340) was from Sigma. The Microplate reader (Multiskan/MCC340) was from Thermo Labsystems (Helsinski, Finland) and distributed by Thermoscientific (Beverly, MA, USA). Precision pipettes with disposable tips, and disposable syringes with 18 G needle were used for convenient removal of supernatants. Centrifugation (12,000× g) was conducted with capped polypropylene vials (1.5 or 2 mL size) of Eppendorf type. The Microplate reader used was a Multiskan/MCC340 from Thermo Labsystems (or any spectrophotometer with 600–620 nm filter will do).

Preparation of Dried Collagenous Biomaterial (BCD)

One half of a bovine hide sample was treated traditionally from dehairing to deliming stage (Aldema-Ramos *et al* 2008 and 2010). The hide was pickled to pH 3.5 with ~1.5% formic acid then washed two times with 200% (w/w) water for 20 min (Castell *et al*, 2009a and 2009b). In the pickle stage, the side was sammed and cut into five strips as indicated in Figure 2.

Figure 2. How five strips of hides were taken for further experimentation



Decorin Analysis Based on sGAG Portion of the Molecule

Alcian Blue stock solution (3 mg/mL) containing 0.1% H_2SO_4 and 0.4 M GuHCl (Guanidine-hydrochloride) was prepared. The final concentration of Alcian blue used in each assay solution was about 0.15 mg/mL. Aliquots of 8M GuHCl were used to dilute the samples to a final concentration of 0.4 M in the assay solution. The solution used in diluting the Alcian Blue stock solution and as addition to samples contained 0.3% H_2SO_4 and 0.75% Triton X-100. The DMSO (dimethylsulphoxide) solution used in washing off contaminations and interfering matrices in pellets was composed of ~ 40% DMSO and 0.05 M $MgCl_2$. Gu-Prop solution containing 4 M GuHCl, 33% 1-propanol and 0.25% Triton X-100 is used to dissolve bluish pellets before reading their absorbance at 605 nm (Kamiya SGAG kit manual 2006).

The Near Infrared Spectroscopy

The chemical structure at the surface of the BCD sample was analyzed by utilizing near InfraRed (NIR) Spectroscopy (Barton 2002). The NIR signals are due to the excitation of vibrational modes within molecules where the strong and significant signals correspond to functional groups with dipole moments containing NH, OH or CH bonds (Workman and Weyer 2008). The NIR measurements do not require rigorous sample preparation, especially with the robust handheld NIR instrument such as Phazir from Thermo Fisher Scientific, and still capable of giving valuable information (Microphazir manual 2011). To determine the differences and/or similarities of the differently treated BCD samples, the Principal Component Analysis (PCA) was utilized (Esbensen and Galadi 2009).

Results and Discussion

Acetone Drying Efficiency

To measure the drying efficiency of acetone on BCD samples, the water content in the acetone washing floats was monitored and analyzed by Karl-Fischer technique (Table 1). Samples that were acetone washed four and five times, 3S and 4S respectively, have almost the same amount of water in the acetone float, implying that there was hardly any water left in the BCD sample

after the fourth acetone wash. About ~1.5 % water is the least amount of water that can be removed starting from almost 25% of water observed in the first acetone wash of sample 0S.

Table 1. Water content in different acetone washings performed within 60 minutes

Sample code	Sample washing / drying treatment	Water content in acetone float (%)
0S	Acetone dehydrated / 1 wash	24.5 ± 0.10
1S	Acetone dehydrated / 2 washes	6.8 ± 0.15
2S	Acetone dehydrated / 3 washes	3.0 ± 0.00
3S	Acetone dehydrated / 4 washes	1.8 ± 0.05
4S	Acetone dehydrated / 5 washes	1.3 ± 0.00
5S	No Acetone dehydrated / Air dried	N/A

Decorin Analysis Based on sGAG Portion of the Molecule

The Alcian Blue colorimetric determination of decorin based on the sGAG portion of the decorin molecule was utilized (Karlsson and Bjornsson 2001).

The ionic bonding between cationic dyes (such as Alcian blue) and the negatively charged sGAG are generally thought to be proportional to the number of negative charges present on the sGAG chain, *i.e.*, both sulfate and carboxyl groups (Mozersky et al 2003; Aldema-Ramos et al 2008). The intensity of absorption of the bluish coloration is directly proportional to the sGAG concentration in the sample.

The concentration of decorin in BCD samples was calculated from the slope of the standard calibration graph (Figure 3) prepared by plotting a straight line relating the absorbance to the series of known amount of standard sGAG.

Figure 3. Standard graph of Decorin, based on known sulfated glycosaminoglycan (sGAG) concentration.

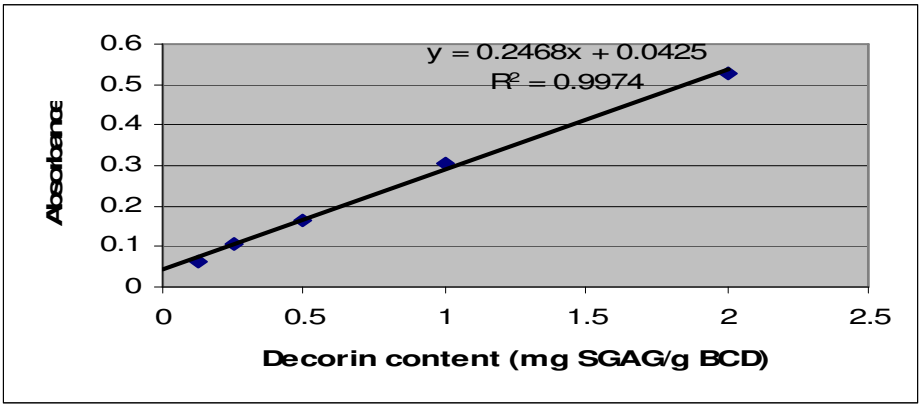
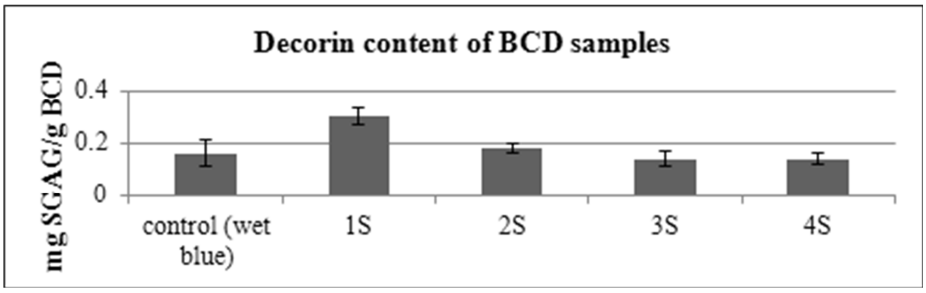


Figure 4. The decorin content of Dried Collagenous Biomaterial (BCD) samples dried different times with acetone.



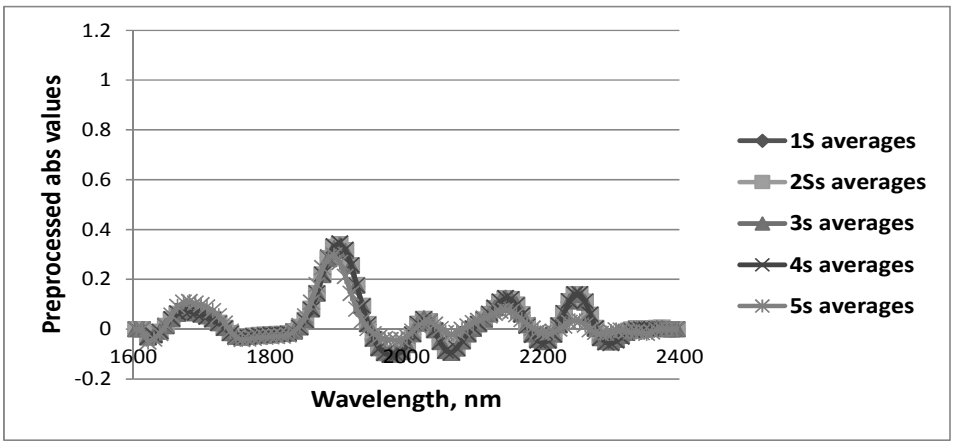
The decorin content of the wet blue obtained from traditionally tanned hide was found to have an average of ~0.16 mg SGAG/g lyophilized hide when analyzed in parallel with BCD samples. Whereas, the BCD samples after fourth washes was about 0.14 mg/g lyophilized BCD sample (Figure 4). The tensile strength and elongation-to-break, among other mechanical properties, measured and previously reported by Aldema-Ramos et al (2012a) have shown that the results are relatively comparable as shown in Table 2. The traditionally tanned leather had almost the same values (with no significant difference) as the BCD sample washed or treated with acetone 4 times (sample 3S). The BCD sample 3S also exhibited similar physical properties in terms of softness, strechability and tensile strength to the traditionally tanned leather. As the number of acetone washes increases, the amount of decorin decreases but after the 4th acetone wash, the decorin removal has reached its optimum value of ~ o.14 mg sGAG/g BDC reminiscent of the traditionally tanned leather.

Table 2. Correlation of decorin content to the mechanical properties of the BCD samples.

Sample Code	Sample Acetone Drying Treatment	Elongation-to-break (%)	Tensile Strength (N/m²)	mgSGAG/g BCD
control (wet blue)	traditional tanning	83	411	0.16 ± 0.05
1S	2 acetone washes	67	386	0.30 ± 0.07
2S	3 acetone washes	68	369	0.18 ± 0.04
3S	4 acetone washes	86	405	0.14 ± 0.05
4S	5 acetone washes	76	361	0.14 ± 0.06

The Near Infrared (NIR) spectra of different BCD (outside or grain) samples that were plotted in absorbance as Log(1/R) or Log(absorbance)$^{-1}$ at each wavelength showed no significant difference. More complete data reported in (Aldema-Ramos 2012a).

Figure 5. Preprocessed (SNV, Savitsky-Golay (1-7-2)) NIR spectra of the samples. Plot shows the preprocessed absorbances at each wavelength.



There are no distinct spectral features to differentiate acetone treated/dried BCD samples 1S to 4S from each other. The air dried or untreated sample 5S however, exhibited slight peak shifts and height differences such as at 1682nm, 1894.2nm, 2033.7nm, and 2246 nm compared to 1673nm, 1902.3nm, 2025.6nm, and 2254nm, respectively for acetone treated samples 1S to 4S samples.

Conclusions

The physical appearance and characteristics of the samples that were dehydrated three to five times by acetone BCD (samples 2S, 3S and 4S, respectively) were similar. After four acetone treatments a good quality BCD sample was obtained which was comparable to the traditionally tanned leather. The maximum amount of decorin was removed after four acetone washings and no further decorin removal was observed in the sample washed five times with acetone. Both BCD samples (washed 4 and 5 times with acetone) possessed a soft, opaque and firm crust leather texture. The NIR spectra of the differently acetone-treated BCD samples were only slightly differentiable in the PCA cluster plots, or according to sample grouping in the statistical analysis. The broad bands in NIR spectra could arise from the overlapping absorption bands of polar functional groups in different intramolecular and extramolecular environments of the molecules in the sample. Overall, the analysis of inside (corium) *vs*. outside (grain) of the BCD samples generated similar results. The spectral features were super-imposable and not distinguishable from each other. On the other hand, the air dried BCD sample (5S) appeared translucent and was a very hard material. Its NIR spectrum was quite distinct from the spectra of the other four acetone-treated and air-dried samples. This implied that the waterless drying treatment before chrome tanning made the material quite resistant to any harmful effects of the organic solvent. The mechanical properties or quality of BCD samples washed/dried with acetone four to five times were comparable to the traditionally tanned crust leather products. The acetone dehydration technique was quite superior to the traditional tanning process because almost no waste water was generated by this technique. The process could also be quite economical because the acetone washes can be collected, recovered and recycled.

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