

IDENTIFICATION OF DECORIN AND OTHER PROTEINS IN BOVINE HIDE DURING ITS PROCESSING INTO LEATHER

by

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ABSTRACT

The present work attempts to develop a more accurate and reliable assay for decorin concentration in hides as they are processed to leather. The ELISA technique previously developed for decorin analysis is further improved and optimized by treating the samples with chondroitinase-ABC after dialyzing them in the presence of collagenase. Some newer techniques developed and adapted include SDS-PAGE and Western blotting using immuno-colorimetric staining. Probing the blotted protein with a combination of two different antibodies specific for decorin, i.e., PK1 and 6D6, is more specific and sensitive than using either antibody individually. Western Blotting can be used visually and quantitatively to confirm that ELISA-detected species are indeed decorin. The intact proteoglycan as well as the core protein and resulting fragmentations of decorin are determined after treating the samples with collagenase and chondroitinase ABC alone or a combination of these two enzymes. The fate of the different proteins in raw hides to leather is followed by analyzing the separated protein bands in SDS-PAGE gel using MALDI-TOF peptide mapping.

RESUMEN

El presente trabajo pretende desarrollar un cateo más preciso y fiable de la determinación de la concentración de decorin en pieles que son transformadas a cuero. La técnica ELISA desarrollada previamente para el análisis de decorin es mejorada y optimizada tratando las muestras con Condroitinasa-ABC luego de dializarlas en presencia de colagenasa. Algunas nuevas técnicas desarrolladas y adaptadas incluyen electroforesis del gel de la poliacrilamida del sulfato deodecil de sodio (SDS-PAGE) y el método Western Blot utilizando tintados inmuno-colorimétricas. Probando la proteína tintada con una combinación de dos anticuerpos específicos para decorin, por ejemplo, 6D6 y PK1, es más específico y sensible que el uso de anticuerpos en forma individual. Western Blot pueden utilizarse visual y cuantitativamente para confirmar que la especie detectada por ELISA son realmente decorin. Los proteoglicanos intactos como así el núcleo de proteínas y la consiguiente fragmentación de decorin se determinan después de tratar las muestras con colagenasa y Condroitinasa-ABC, solo o en una combinación de estas dos enzimas. El destino de las diferentes proteínas en las pieles crudas hacia el cuero es seguido por el análisis de las bandas de proteínas separadas en gel SDS-PAGE usando un mapeo de los péptidos por MALDI-TOF.

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INTRODUCTION

The different sections of raw hides are observed to have significantly varying amounts of initial decorin content. When each hide part was subjected to the same tanning treatments, however, the amounts of available residual decorin were found to be significantly the same in all samples⁽¹⁾. Removal of proteoglycans efficiently from the hide, when it is processed into leather, is generally acceptable and beneficial for leather quality, in particular for softness and flexibility^(2,3). Our current efforts are geared toward the removal of the predominant and best understood proteoglycan of skin, decorin^(1,3). Decorin is a small extracellular matrix proteoglycan involved in several fundamental biological functions, including the organization of collagen fibrils in skin⁽⁴⁾. It 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. Most of the protein is comprised of a chain of ten "leucine-rich repeat (LRR)" segments that tend to aggregate with each other and bind probably at two sites at each end of the collagen molecule^(2,3). In nature, the LRR segments adopt a stacked β -sheet/ α -helix hairpin structure resulting in an overall horseshoe shape; that structure tends to dimerize as its stable configuration^(4,6).

MATERIALS AND METHODS

Sample Preparation and Extraction

The detailed procedure of sample preparations, tanning treatments, protein extraction, and dialysis were illustrated in a previous report⁽¹⁾. Procedures for in-gel digestion of peptides from SDS-PAGE gels were done according to Promega Technical Bulletin No. 309.

Sample Concentration

Precipitating the samples with trichloroacetic acid (TCA) allowed us to concentrate them 20-100 fold, thus allowing us to load a reasonable amount in each gel lane for valid quantification. TCA precipitation can free the protein sample from the other contaminating or interfering materials to give a relatively uniform background and to reduce gel-like properties of the sample solution for easy handling during SDS-PAGE gel loading.

Precipitation was performed by placing a 0.65 ml aliquot from the raw hide extract into an Eppendorf tube. To attain the final concentration of 10% TCA, 71.5 μ l of 100% TCA was added to each tube. For the other samples (dehaired, relimed, delimed and bated), two 1.3-ml aliquots were used and 143 μ l of 100% TCA was added to each tube. For all samples, except raw hide, a 5 μ l of 5% Na-deoxycholate was added to enhance protein precipitation. All samples were mixed well and incubated over ice for 15 minutes. The precipitated protein was obtained by spinning the tubes at

high speed in a microfuge for 15 minutes (in a cold box). The supernatant was carefully removed and discarded. The tube was washed with 0.5 ml 10% TCA. At this step, the two 1.3-ml aliquots were carefully combined to give total of 2.6 ml for each of the dehaired, relimed, delimed and bated hide samples. These samples had 1.0 ml 10% TCA total washings added to them and the supernates were carefully removed after spinning the tubes again. To obtain sharp bands of ~1-10 μ g protein per lane, the precipitated sample was resuspended and solubilized by sonication using a microprobe after adding ~100 μ l of SDS-PAGE reducing loading buffer (X1) to the raw hide sample, whereas only ~40 μ l was added to all the other hide samples.

SDS-Polyacrylamide Gel Electrophoresis

Running an SDS-PAGE gel showed relative amounts of the different proteins present in each sample. The optimum amount of 20U collagenase during dialysis was found to effectively remove the stickiness or high consistency of the samples due to collagen so the samples could be handled easily during SDS-PAGE gel loading⁽¹⁾. Precast gels of 12 % Tris-HCl from BioRad were used. The resolution of the protein bands was further improved by using Tris-Tricine instead of Tris-Glycine as running buffer. Another way of improving the identification of different forms of decorin was by treating the sample with chondroitinase-ABC to remove the glycan tail, which may be the cause of the smeary band at ~100kDa.

The protein extracted from raw and dehaired hides was treated in three parallel ways. The first was dialyzed with collagenase overnight in PBS buffer, incubated for 1 h at 37°C and then heated for 5 min at 100°C to inactivate the enzyme. The second sample was dialyzed overnight in PBS buffer without collagenase, incubated for 1 h with chondroitinase-ABC at 37°C, and then heated for 5 minutes at 100°C to inactivate the enzyme. The third sample was dialyzed with collagenase overnight in PBS buffer, incubated for 1 h at 37°C with chondroitinase-ABC, and then heat-inactivated for 5 min at 100°C.

Western Blotting

The electrophoretically separated proteins in SDS-PAGE gel were Western-blotted onto an Immuno-Blot polyvinylidene-difluoride (PVDF) membrane (from BIORAD; Catalog 162-0175, 0.2 μ m). In the Western Blotting procedure followed in this work, the blotted membrane was first exposed to the first primary antibody PK1, for at least 1 h. PK-1 is a rabbit anti bovine decorin antibody (prepared by Alpha Diagnostics International, San Antonio, Texas) that recognizes the 16-amino acid sequence at the N-terminus of bovine decorin. The same membrane was then incubated for another hour with a second primary antibody, 6D6 (courtesy of Dr. Paul Scott, University of Alberta, Canada). The 6D6 antibody recognizes a sequence of 4 amino acids at the C-terminus (residues 241-244) of decorin. The excess primary antibodies were removed by washing the membrane three times with washing buffer. The

secondary antibodies, anti mouse-HRP against 6D6 and an anti rabbit-HRP against PK1, were added simultaneously and allowed to incubate at room temperature for another hour with shaking. The excess secondary antibodies were removed by washing the membrane with washing buffer. Finally, a colorimetric peroxidase reaction was induced by adding 3,3',5,5'-Tetramethylbenzidine (TMB) substrate and incubating for a few (~15-30) minutes until a visible deep purple color was observed. Water was added to stop further reaction. The blotted membrane was then scanned using a Personal densitometer from Molecular Dynamics and the different bands were assayed for their corresponding molecular weights using the Image QuANT and quantified using the Image-J program from NIH. TMB is part of the Protein Detector™ Western Blot Kit TMB system, product code 54-11-50 (KPL, Gaithersburg, MD).

Protein Confirmation

MALDI/I mass spectrometry using automated tandem time of flight fragmentation of preselected ions (MALDI/TOF-TOF) has been previously described⁽⁷⁾. Spectra taken on a 4700 Proteomics Analyzer - MALDI-TOF/TOF were analyzed using the MSDB database, available from the National Center for Biotechnology Information (NCBI), with the conditions of 50ppm precursor tolerance, minimum signal to noise ratio of 10, and methionine oxidation variable modification.

RESULTS AND DISCUSSION

An SDS-PAGE gel in Figure 1 is showing a good resolution of relative amounts of decorin and other proteins present in each sample taken from different treatments during tanning of hides to leather. When the method was tried and tested, only the dialyzed samples were successfully loaded to the gel after the gelatinous consistency is removed due to elimination of collagen. The standard decorin band appears smeary with a median size of ~100 kDa, indicating that the heterogeneous glycan chain of varying molecular weight of 20-50KD is still intact. The sharper protein bands running between bovine serum albumin at ~100kDa and carbonic anhydrase at ~37.4 kDa are very visible on lanes 2-4 (L2-4), from raw hide samples taken from butt (L2), belly (L3) and shoulder (L4). However, after dehairing, as shown in lanes 5 to 7 (L5-7), and after reliming, in lanes 8 to 10 (L8-10), those bands are clearly diminished even though the amount of samples loaded in lanes 5 to 10 (L5-10) is about ten fold more concentrated than in lanes 2 to 4 (L2-4) from raw hide samples. It is clear that most of these proteins in raw hides are gone after subjecting them to tanning treatments converting hides to leather. It is interesting to know which of those bands are decorin or decorin-containing fragments by immunoblotting the gel with antibodies particularly specific towards decorin.

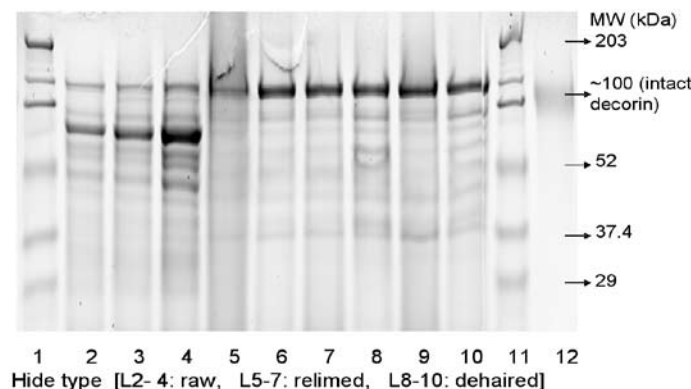


Figure 1: SDS PAGE gel of different hide samples after dialyzing protein extracts in the presence of collagenase. Lanes 1 and 11: broad protein standard ladder from BioRad; Lanes 2-4: 5µg raw hide samples [L2(belly), L3(butt), and L4(shoulder)]; Lanes 5-7: 40 µg relimed hide samples [L5(belly), L6(butt) and L7(shoulder)]; Lanes 8-10: 40 µg dehaired hide samples [L8(belly), L9(butt), and L10(shoulder)]; Lane 12: 2 µg decorin standard (intact).

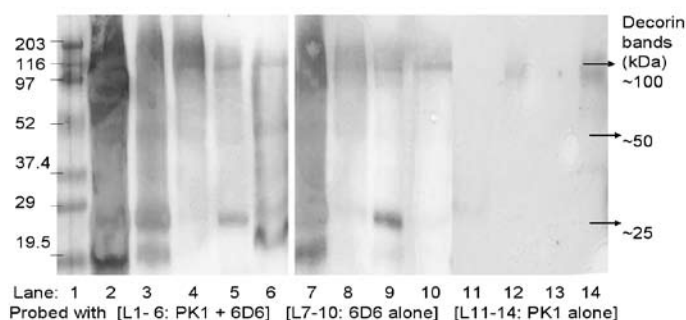


Figure 2: Western blotting of hide samples taken from different standard tanning treatments converting them to leather. Probing with 2 antibodies, PK1 and 6D6, specific for decorin. Lane 1: pre-stained broad protein marker; Lanes 2 and 14: raw hide samples; Lanes 3, 7 and 13: dehaired; Lanes 4, 8 and 12: relimed; Lanes 5, 9 and 11: delimed hide samples; Lanes 6 and 10: bated hide samples. The arrows indicate presence of decorin; at ~100 kDa it is still intact with SGAG chain; at ~50 kDa it is a monomeric core protein; whereas ~25 kDa are decorin fragments where the epitope of 6D6 is still present but that of PK1 in the N-terminus is gone.

The fragmentations were verified by SDS-PAGE and complimented with Western blotting as shown in Figures 2 and 3. Decorin survived the tanning treatment even under conditions that remove the GAG (glycosaminoglycan) chain, as clearly shown by the sharp bands of decorin core protein at ~50KD on SDS-PAGE gel (Figures 1, 2 and 3). The relative amount of decorin shown from the shoulder sample is greater than from the butt and in turn greater than from the belly part of hide, confirming the results previously obtained by ELISA technique⁽¹⁾. Utilizing the combination of two antibodies specific for decorin, 6D6 and PK1 added serially, helped us get a more reliable result. In a parallel trial, where both the antibodies PK1 and 6D6 were added and

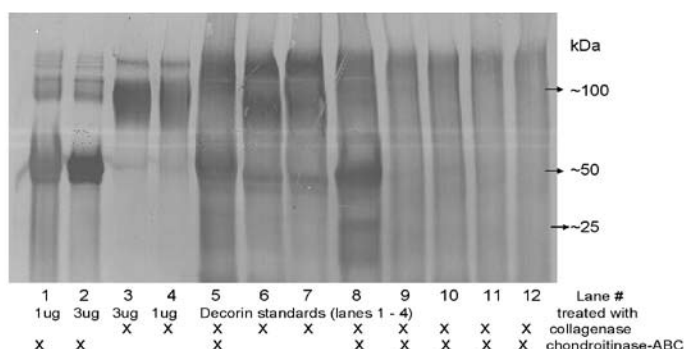


Figure 3: Western blotting of various hide samples treated with collagenase, chondroitinase-ABC, or with both, showing decorin-containing bands. Lanes 1-2: loaded with decorin standard treated with collagenase and chondroitinase-ABC; Lanes 3 to 4: loaded with untreated decorin standard or "as is;" Lanes 5-8: raw hide samples; Lanes 9-10: relimed hides; Lanes 11 and 12: delimed hide samples.

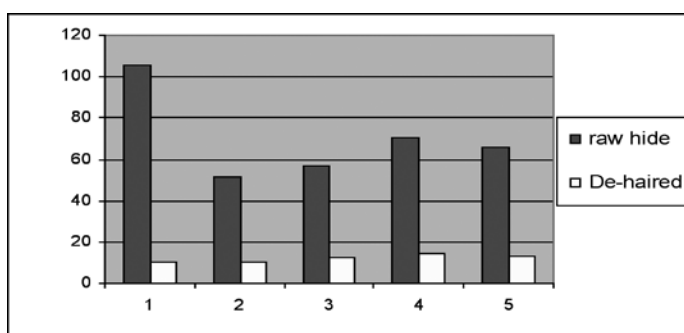


Figure 4: Comparison of available decorin found in differently treated raw and dehaired hide samples using ELISA technique. 1. no treatment; 2. dialyzed with collagenase; 3. dialyzed with collagenase, incubated for 1 h at 37°C, heated to 100°C for 5 min; 4. chondroitinase-ABC was added after dialysis (NO collagenase was added), incubated for 1 h at 37°C, heated to 100°C for 5 min; and 5. dialyzed with collagenase, then treated with chondroitinase-ABC incubated for 1 h at 37°C, then heated to 100°C for 5 min.

simultaneously incubated, the blotted membrane gave similar results. Only decorin and decorin-containing fragmentation products, where the epitope of either PK1 or 6D6 was still intact, gave the deep purple color.

Due to the sensitivity of the Western blotting technique, the bands are showing more clearly and with higher intensity, as seen in Figures 2 and 3. The protein bands running along ~100 kDa are indeed the intact decorin. The protein band that runs along molecular mass of ~50kDa on a 12% SDS-PAGE gel is confirmed to be the core protein on a Western blotted PVDF membrane and after chondroitinase-ABC treatment. The anomalous migration of proteoglycans on SDS-PAGE giving a smeary band along ~100kDa could be due to the microheterogeneity in disaccharide composition of the SGAG chain still attached and can affect the total charge density of the molecule. After chondroitinase-ABC treatment, a sharper core protein band of the standard

decorin loaded on lanes 1 and 2 of Figure 3 is observed running along ~50kDa MW region. Chondroitinase-ABC removed the heterogeneous glycan tail^(5,6).

After the deliming stage of tanning hides to leather, the ~25kDa band is clearly probed with either both 6D6 and PK1 (L5 of Figure 2) or 6D6 alone (L9 of Figure 2), signifying that this is also a fragment of decorin where most of the N-terminus, including the epitope for PK1, is missing.

As previously reported^(1,5), the amount of decorin removed from the hide during dehairing did not change much in succeeding tanning steps. The concentration of available decorin in dehaired hide samples is almost the same as the concentration available from bated (final step before chrome tanning to leather) samples, whether the decorin extract is dialyzed or not. Therefore, it is justifiable to take raw and dehaired hides as representative samples in the dual enzyme treatment of collagenase and chondroitinase-ABC to monitor the relative changes of decorin and other proteins present in raw hides as they are subjected to tanning treatments. The summary of the results is graphically shown in Figure 4. The graph shows that the undialyzed raw hide sample (black, bar1) has the highest amount of available decorin content (~10.0 µg/g decorin/g hide) detectable by ELISA technique. Once the samples are dialyzed and treated with collagenase (black, bar 2) or chondroitinase-ABC alone (black, bar 3), the amounts of available decorin tend to drop to about 50% from the undialyzed sample to merely ~50 µg decorin/g hide. When the hide sample is treated in series with collagenase and then chondroitinase-ABC, the values tend to increase a bit by ~18% to an average of 65 µg decorin/g hide (black, bars 4 and 5). However, for the dehaired hide sample (white bars), no matter how one treats the sample; either with or without collagenase and chondroitinase-ABC, the decorin content seems to remain the same at ~12 µg decorin/g hide.

CONCLUSION

SDS-PAGE or polyacrylamide gel electrophoresis and Western blotting can be used to confirm that the previously ELISA-detected species are indeed decorin and that it is a combination of intact (at ~100kDa) and core protein (at ~50kDa). However, the fragmentation product at ~25kDa may not be detected by ELISA due to the missing epitope for the antibody PK1 that anchors the decorin to the microplate well. Probing the blotted protein with a combination of two different antibodies specific for decorin, PK1 and 6D6, at the same time is more specific and sensitive than using either single antibody. The anomalous migration of proteoglycans on SDS-PAGE appearing as a smeary band can be due to the microheterogeneity in disaccharide composition of the SGAG chain still attached, which has affected the total charge density of the molecule⁽⁶⁾.

TABLE I

Different proteins identified in raw hides but substantially diminished or removed in tanned hide samples using SDS-PAGE and MALD/I TOF-TOF mass spectrometry

Identified Proteins in Raw and tanned hides	Accession #	MW (Da)	Protein band at kDa region
Bovalbumin	AAN17824	69248	~130
BSA precursor	ABBOS	69225	
Bos taurus fragment	AF542068	69278	~100
Decorin-Bos taurus (dimeric core and intact decorin protein)	Q3MHN1_BOVN PGS2_BOVN	39854 39947	~100 and 50
Decorin precursor (monomeric core)	S06280/AF125041	39812	~50
Bos taurus fragment and Hemoglobin subunit β -A chain (in raw hide only)	BAC56541 P04346	14383 15954	~25
Bos taurus fragment and Histone H4 (in raw hide only)	BAC56404 H4_BOVN	11949 11229	~15

Knowing exactly what are the different proteins present in the raw hide and which are left after the different treatments involved in tanning hides to leather is quite challenging since the amounts of the extracted proteins left after tanning treatments are too low to get reliable results. These newly optimized analytical techniques can be a better way of analyzing the different tanning treatments, especially if the blotting is made linear and directly proportional to the amount of the protein loaded on each lane on the gel.

Having a more reliable analytical technique will enable us to find out what is the optimum amount of decorin that could be allowed to remain in the finished leather to account for soft, flexible, and yet strong characteristics. Later, application of this knowledge will assist us in developing and incorporating enzyme treatment systems to obtain leathers with better properties than leather from conventionally tanned hides.

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