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Persistence of Minority Macromolecules of Hide Through the Beamhouse. III. Persistence of Decorin*

by

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Abstract

Decorin, a small dermatan-sulfate proteoglycan that is located on the surfaces of the collagen fibrils of hide and skin, is a minority macromolecule that might play a role in leather drying. We show by immunohistological techniques that not even the amino terminal of the molecule, which is not bound directly to collagen, is removed in the beamhouse. Unlike collagens type VI and XIV, it was not found in the grain enamel. Residual decorin might be an important feature of hide and grain quality in leather.

Introduction

Retans and fatliquors are added to leather to control the adhesions among its fibers, but it is not clear why fibrils adhere to each other, nor whether they actually would adhere if their surfaces comprised only type-I collagen. Besides collagen type-I, the fibrils of hide contain proteoglycans and non-fibrillar collagens. Although present in small amounts, they are concentrated on the surfaces of the fibrils and probably enhance the tendency of the fibers and fibrils of leather to clump when it is dried. It is therefore necessary to determine their role if fiber adhesion during drying is to be understood. In previous publications we reported on the removal or persistence of minority collagens III,[1] VI, XII,[2] and XIV[1] during preparation of hide for tanning; here we report on the proteoglycan decorin.

Decorin is a small proteoglycan found in association with collagen fibrils in the extracellular matrix of, *inter alia*, skin, bone and cartilage. It consists of a core protein of 329 amino acid residues,[3] M_r = 43,000, and a single dermatan sulfate (DS) side chain, M_r = 16,000, attached to the amino terminal.[4] The core protein contains two binding sites to the α-chain of type I collagen, located between amino acids 15-125 and 185-329, respectively.[5] The partial removal of the dermatan-sulfate side chain in the beamhouse was described earlier.[6] Although enzymes are effective in removing it, most of the mass of the proteoglycan is in the protein part, whose fate has not yet been investigated.

Materials and Methods

Bovine Hide

Two fresh Black Angus hides, procured the same day, were each cut into their two sides, which were labeled 1 or 2 from hide A, and 3 or 4 from hide B. Samples for immunohistochemical analysis were excised, from the neck, rump and belly areas, immediately following each leather-process step of fleshing, relime and bate.

Enzymes

Purified subtilisin Carlsberg (E. C. 3.4.21.14) without additives (Proteases B and C, Novo, Franklinton, NC) was used, Protease B for side 1 in the relime; Protease C for side 3 in the bate (*vide infra* Leather Process). The porcine pancreatic enzyme used as a conventional bate was Rohapon 6000 (Rohmtech, Inc.).

Antibodies

PK-1, a specific anti-(bovine decorin) directed against the 16 amino acids of the N-terminal (DEASGIGPEEHF-PEVC),[7] was prepared in rabbits by Alpha Diagnostics International (San Antonio, TX), who also synthesized the peptide. The specificity of the antibody PK-1 was correlated with samples of an anti-bovine decorin antibody (LF-94) obtained from Dr. Larry W. Fisher of the National Institute of Dental Research, National Institutes of Health. There was no noticeable difference in results between the two anti-decorin preparations, and no nonspecific binding by PK-1.

Leathermaking Process

The "unmodified process" was the standard USDA process,[8] which was used for sides Nos. 2 and 4. Variations were examined in the reliming and bating steps for sides 1 and 2, as shown in Figure 1. Note that the USDA relime bath contains a small amount (1%) of NaHS. Samples of hide were taken during the process to perform matched trials as follows:

- unmodified lime vs lime with subtilisin
- unmodified bate vs bate with subtilisin
- lime with subtilisin followed by standard bate vs standard lime and standard bate.

In these experiments, side 2 served as the control for reliming; side 4, for bating. Although the relime step includes reduction with sodium hydrosulfide, this reagent does not react with subtilisin, which contains no cysteine or cystine.

Immunohistochemistry

The details of our procedures are described in reference (1). Sample blocks 3 x 5 x 4 mm were removed from the preserved specimens and were prepared for sectioning by snap-freezing in liquid nitrogen. Full cross sections of hide were cut 8-10 microns thick at -28°C with a freezing microtome and were stained using the peroxidase-antiperoxidase method for development of a brown color in the areas where decorin protein was present.

Microphotography was accomplished with an Olympus BX50 System with an SC35 camera attachment. Stained sections were photographed using ISO 25 Kodak Royal Gold fine-grain film under constant light and magnification settings. Goat anti-rabbit immunoglobulin (Boehringer Mannheim Corporation, Indianapolis, IN) and rabbit peroxidase anti-peroxidase (PAP)(Cappel Laboratories, Durham, NC) were used as the secondary and tertiary antibodies, respectively. Immunohistological detection was accomplished using Sigma Co. (St. Louis, MO) Fast DAB Tablet sets.

Mechanical Testing

Following chrome tanning, all four sides were retanned, fatliquored and colored together (Figure 5). Ten samples were die-cut from the butt area of each side and were examined with a tensile tester (Instron Model 1122, Instron Corp., Canton, MA) for mechanical properties following ASTM Standard D2209-95.

	HIDE A		HIDE B	
	Side 1	Side 2	Side 3	Side 4
Relime	Lime 2% NaHS 1% Protease B 0.12% 16 hours	Lime 2% NaHS 1% 20 hours	Lime 2% NaHS 1% 20 hours	Lime 2% NaHS 1% 20 hours
Bate	$(NH_4)_2SO_4$ 3% Boron TS .10% Rohapon 6000 0.15% 90 minutes	$(NH_4)_2SO_4$ 3% Boron TS .10% Rohapon 6000 0.15% 90 minutes	$(NH_4)_2SO_4$ 3% Boron TS .10% Protease C 0.015% 60 minutes	$(NH_4)_2SO_4$ 3% Boron TS .10% Rohapon 6000 0.15% 90 minutes

FIGURE 1. — Treatment of sides for analysis of effects of beamhouse treatments.

RESULTS

Decorin is associated with all the fibers throughout the fresh hides (Figures 2A, 2B). It is seen in Figure 2A that in the corium minor of fresh hide A, decorin could not be detected in the nonfibrous parts, neither in the epidermis, hair follicles, nor blood vessels, which were unstained with anti-decorin. Although the staining is not uniform among the fibers, there are no obvious systematic concentrations. The results from all the sides are similar (not shown).

Some blackening was observed in these tissues due to endogenous peroxidases (including methemoglobin in the coagulated blood). This artifact is even seen in the sample of hide A that was stained without the primary antibody (anti-decorin) (Figure 2C); a colored product is generated at some locations in the epidermis and at hair follicles even though there is nothing to bind the horseradish peroxidase. The color in this control sample, however, is deep black instead of the reddish-brown of labeled fibers. The fibers in Figure 2C are unstained and are hardly visible, showing the selectivity of the development system for anti-decorin used in these experiments - the secondary antibody binds to anti-decorin but not to the bovine tissue.

Unlike in the staining of collagen XIV shown earlier,[1] there is in Figure 2A no specifically stained dark band underneath the epidermis that would correspond to the grain enamel in the eventual leather. The epidermal basal layer forms a line of cells that are stained black, appearing as a line of dots, while the stratum corneum above it is colorless.

The standard[8] relime, which includes sodium hydrosulfide (Figure 1), removed the epidermis and blood vessel walls (Figure 3). The staining of the fibers due to decorin protein, however, is no different from that in fresh hide (cf. Figure 3A and Figure 2A). We conclude that limehydrosulfide does not remove the amino terminal of the decorin protein from the fibers, although it might denature it.

The side that was treated with lime (hydrosulfide) plus protease is indistinguishable from that treated with lime (hydrosulfide) alone (cf. Figures 3B vs 3A). The line of basal cells from the epidermis persists, at least partially, in both samples from the reliming.

The bating enzymes removed all remnant epidermis, including what we identified as basal cells (Figure 4). There is, however, no apparent removal of decorin protein, neither by treatment with pancreatic protease (Figures 4A, 4B) nor by subtilisin (Figure 4C). Nor did pre-treatment with subtilisin in lime make any difference (Figure 4D) in the later effect of the pancreatic bate. The fibers of the corium major are thicker than those in the corium minor, but the persistence of decorin protein is similar (Figure 4B).

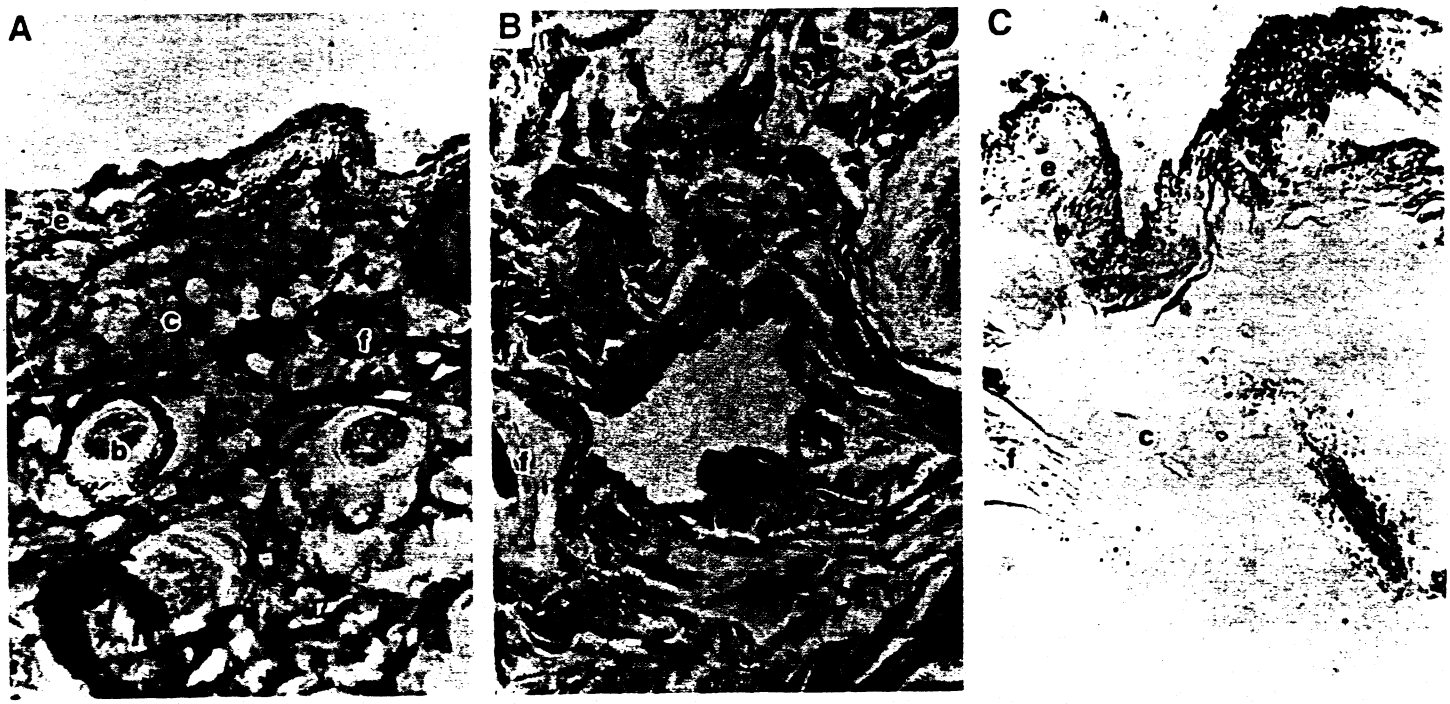

FIGURE 2. — Staining of fresh hides by anti-decorin. (A) corium minor; (B) corium major; (C) control corium minor, developed by usual procedure but without anti-decorin.
c = corium minor; e = epidermis; f = hair follicles.

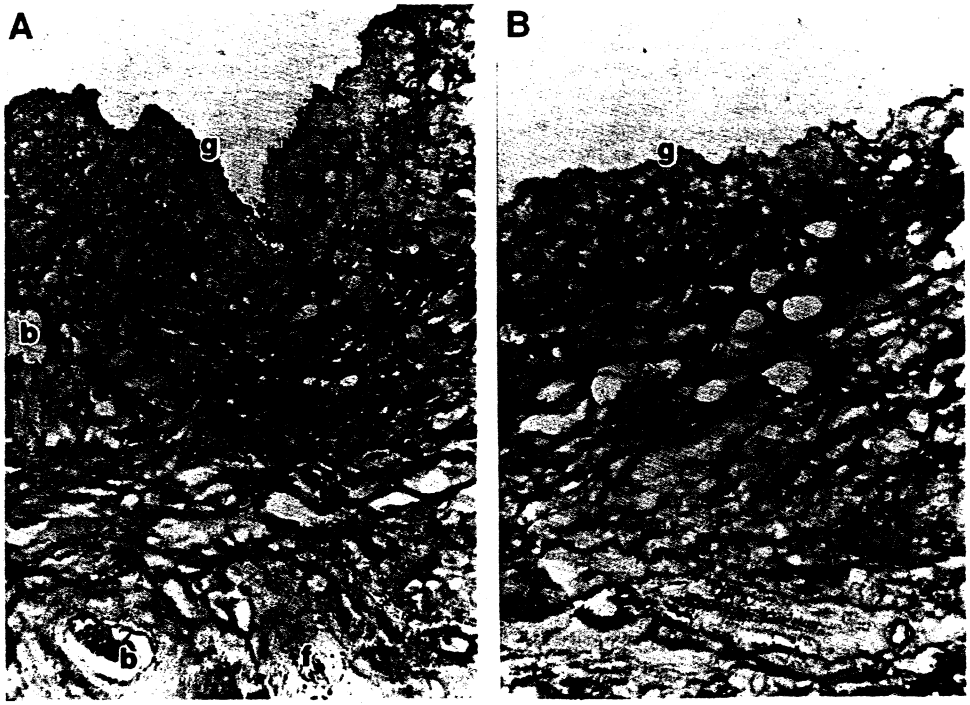

FIGURE 3. — Staining of fresh hides by anti-decorin after relime. (A) unmodified lime process. (B) lime with subtilisin. c = corium minor; f = hair follicles; g = grain enamel.

The different treatments gave no significant variations in tensile properties of the sides, measured after they were tanned, dried, and finished (Table I). The strain and stress at break contribute to the energy at break, which is related to the toughness of the leather. The secant modulus (the slope of the initial part of the stress-strain curve) describes the stiffness.

Discussion

In the beamhouse, a hide is subjected to a very drastic chemical process, designed to remove the epidermis, hair, cells, nerves, and muscles. It reduces disulfide bonds of proteins, by which reaction the epidermis and hair are removed. The lime chemically alters the collagen, hydrolyzing some of the amides of asparagine, and cleaves much of the glycosaminoglycan (GAG). The mixture of hydrolases in the bate attacks most of the proteins, although collagen triple helices are resistant.

To determine which macromolecules survive the beamhouse, antibody staining has been used.[1,2,9] This method is definitive if used with selective polyclonal antibodies against epitopes distributed over the whole macromolecule. When monoclonal antibodies are used, however, only the fate of the part of the macromolecule that contains the single epitope is found. Thus, Kronick *et al.*[2] could only state that the NCl segment of collagen XII and the NC3 segment of collagen XIV persisted, while the persistence of collagen VI until the bate was shown by a polyclonal antibody. Similarly, Stephens *et al.*[9] could state that part of the collagen VII molecule persisted at the location of the grain enamel through the bate, being removed by alkaline protease, and that part of the laminin molecule, located similarly, was removed by the bate. In general, the triple-helical segments of collagen molecules are not very immunogenic, so do not react strongly with antibodies.

Assuming, however, that none of these proteins are cleaved in the beamhouse, one can state that we now know that the fibrous collagens I and III persist, but the minority collagens IV, VI, XII, and XIV are removed before tanning. Of these, collagens XII and XIV are found at the surfaces of the fibrils in hide.

Decorin is an adhesion proteoglycan that is also associated with the surfaces of collagen fibrils.[10] The single molecules appear in the electron microscope as horseshoe-shaped bodies (protein)[11] attached to thin tails (DS). Most of the protein comprises a chain of ten "leucine-rich repeat" segments that tend to aggregate with each other and bind probably at two sites at each end of the collagen molecule.[10] This tendency to aggregate is lost when the disulfide crosslinks of decorin are cleaved by reduction and alkylation, causing the denaturation temperature to fall from 43°C

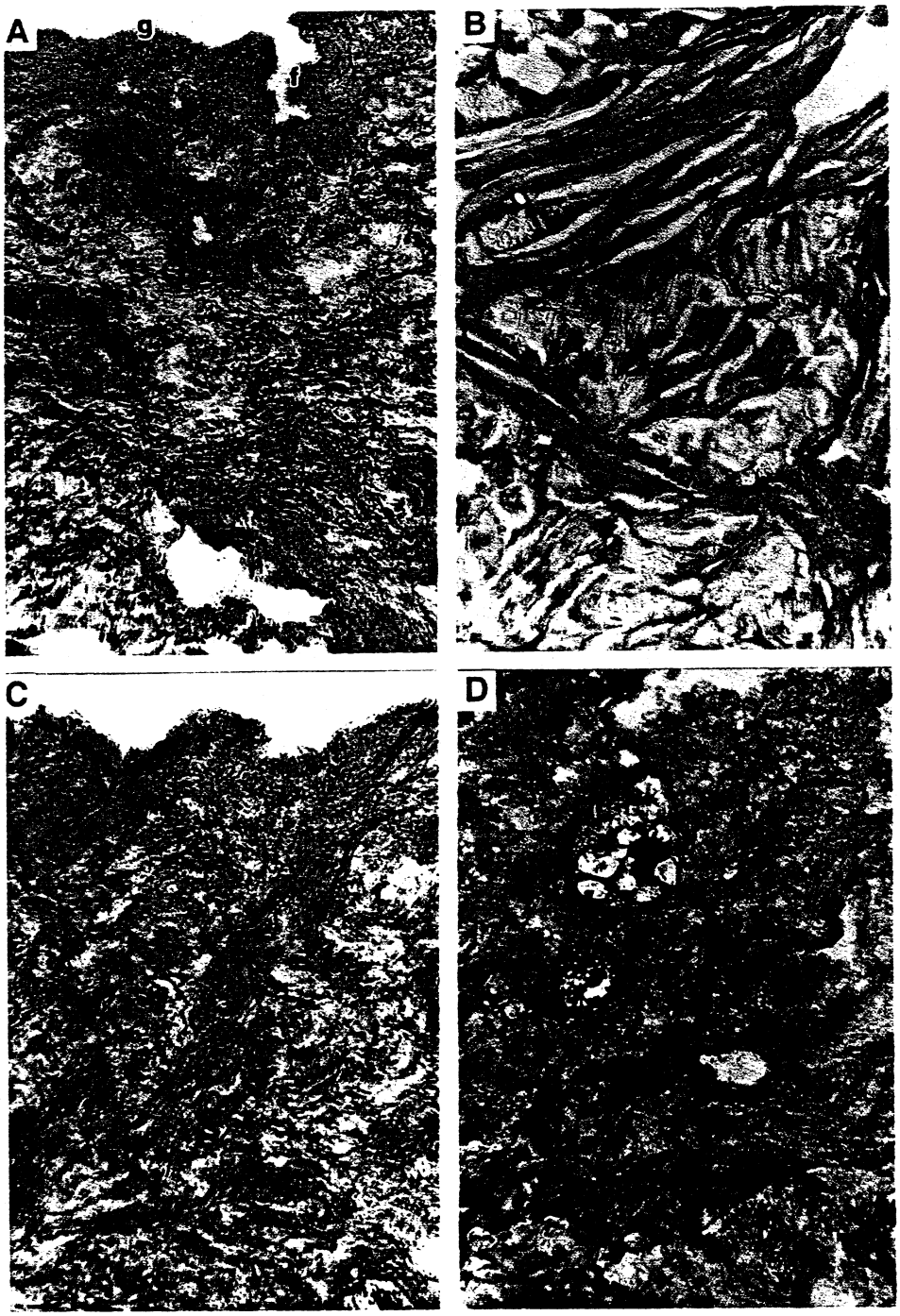

FIGURE 4. — Staining of fresh hides by anti-decorin after bates. (A) corium minor after pancreatic protease; (B) corium major after pancreatic protease. (C) corium minor after bacterial bate (subtilisin). (D) corium minor after lime containing subtilisin followed by pancreatic bate. f = hair follicles; g = grain enamel.

SPLIT WEIGHT FOR PROCESSING 34.60 lb

Wash with running water at 100°F for 10 minutes. Drain, proceed with neutralization.

NEUTRALIZATION

Float	100%	4.17	gal
Sodium bicarbonate	1.25%	0.43	lb
Water	25%	1.04	gal

Run 60 min @ 16 RPM, 100°F pH 5.0.

Wash 5 min with 100% float at 110°F.

Drain float.

RETAN/COLOR

Float	100%	3.13	gal
DLE Syntan	10%	3.46	lb
Baysyntan NNOL	2%	0.69	lb
DYE - Acid or Direct, 1%	25%	1.04	gal

Run 45 min @ 16 RPM, 110°F.

Formic acid	0.5%	0.17	lb

Run 45 min @ 16 RPM.

Batch-wash three times with 100% Float @ 130°F.
Drain completely.

FATLIQUOR

Refloat in 75% water @ 130°F and run drum at 16 RPM

Float	75%	3.13	gal
Altasol CAM - Sulfated oil	5%	1.73	lb
Eureka 400 R - Bisulfited oil	1%	0.35	lb
Water	25%	1.04	gal

Run 30 min @16 RPM.
Add topping oil in 10% water at 130°F

Altasol 310 L - Cationic oil	2%	0.69	lb
Water	10%	0.42	gal

Run 10 minutes.

FIGURE 5. — Standard USDA retan-color- fatliquor process.

TABLE I

Mechanical Properties of Leather Prepared by Various Beamhouse Treatments

	Strain At Break (%)	Stress At Break (kg/mm^2)	Energy to Break Point (kg-mm)	Secant Modulus (kg/mm^2)
Hide A Side 1				
Mean	103.10	3.545	2035.0	1.1870
Std. Dev.	5.69	.337	280.0	.3267
Hide A Side 2				
Mean	92.51	3.257	1748.0	1.1150
Std. Dev.	10.29	.442	230.0	.1589
Hide B Side 3				
Mean	112.90	2.481	1232.0	.9534
Std. Dev.	8.22	.388	250.9	.2038
Hide B Side 4				
Mean	80.42	2.908	1163.0	1.6020
Std. Dev.	10.51	.503	335.0	.3616

to 27°C. The unhairing step breaks disulfide bonds by reducing them with SH^-. At the prolonged 28°C temperature one might expect then that the decorin would become denatured, at least until the reductant is removed. In any case, the decorin protein still binds the collagen. The pentadecameric polypeptide against which the antibody had been prepared would not be expected to fold regularly because of its small length, so would not lose antigenicity on denaturation.

We find it surprising that the decorin protein persists against the attack of pancreatic proteases and of subtilisin. The GAG part can be isolated from the proteins of skin by their proteolysis with papain, and reduced and alkylated decorin can be cleaved into a dozen polypeptides with trypsin,[12] which is the principal enzyme of pancreatic bate. The state of reduction of the decorin in our system after it has been neutralized is not known, nor is its resistance in solution to trypsin. Our observations are consistent, however, with cleavage of decorin protein during bating with persistent association of the amino-terminal fragment with collagen. The epitope of the protein that we used for its detection does not bind collagen,[12] a binding site being located, however, in the adjacent 110-amino acid segment. It does seem possible that, in the densely folded cylindrical native decorin molecule, enough trypsin-sensitive sites could be hidden from the bating enzymes to bind the antigenic N-terminal to the collagen binding site.

CONCLUSIONS

We conclude that collagen fibers of bovine hide are associated with decorin throughout its thickness, and that the protein part of the proteoglycan survives the beamhouse even under conditions that remove the GAG chain. This surviving component might act as an interfibrillar adhesive when the leather is dried.

ACKNOWLEDGEMENTS

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Discussion

Steve Yanek, Buckman Laboratories: It was a great paper. In the immunoassay that you did where you were preparing the two pictures of the ones treated with enzyme or bate, did you conclude now that the pancreatic or the bacterial, either one, had no effect on the decorin or was there some effect? What was your opinion on that?

Susan Iandola: I think that the enzymes are removing the dermatan sulfate side chains and the collagen type XIV protein which I talked about last year, since it is not as helical as collagen type I. It probably removes that also. So you are removing items with the enzyme preparation. All that I am saying is that you are not removing the decorin core protein.

Steve Yanek: The dermatan sulfate is removed but the decorin is not?

Susan Iandola: Right. Your are removing that side chain. So it is doing, I think, what you want it to do. I found the pancreatic porcine bate and the bacterial subtilisin bate to work equally well. They worked equally well based on the decorin and on the quality of the leather as revealed by the tensile strength tests. We retanned, colored, and fatliquored, and the quality of the leather seemed equal in those cases.

Ann Stanley, BLC: I think that this is really an excellent piece of work and really compliments work that we were doing a long time ago and brings it right up to date. A couple of interesting things. The enzymes that you use, the protease, actually degrade dermatan sulfate so one of the things that we now need to find out is what happens in order to release them. We do know that if you use enzymes, the dermatan sulfate removal does happen because we measured it in auditing conditions. So I think that it would be very interesting to combine the measurement of dermatan sulfate removal and the residual decorin and actually try to understand what is going on. I think that this is excellent work and hope that we can collaborate in the future.

Susan Iandola: Thank you Ann.