ESTIMATION OF THE SULFATED GLYCOSAMINOGLYCAN CONTENT OF BOVINE SKIN WITH ALCIAN BLUE*

by

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ABSTRACT

Procedures selected from the work of Bjornsson and Karlsson[1,2] for the quantitation of sulfated glycosaminoglycans with Alcian Blue were modified and adapted to the measurement of this type of polysaccharide in bovine skin. Modifications include: (1) pulverization of the skin under liquid nitrogen, (2) brief, gentle digestion of the skin with collagenase, (3) the use of a variety of Alcian Blue widely used in histologic work, (4) the recipe for the preparation of the Alcian Blue reagent solution, (5) determination of suitable sample size, volumes of reagent solutions, incubation times, duration of centrifugation, etc. The procedure developed yields linear results for 50-300 mg samples of skin; the relative standard deviation for the procedure is ±15%.

INTRODUCTION

In connection with an effort to achieve a better understanding of the role that minority components of the hide play in leather making, we have been considering the possible benefit to leather quality of removing decorin and other proteoglycans from the hide while it is being processed into leather. Alexander et al.[3] showed that liming in the presence of alkaline protease removes some dermatan sulfate from the hide and that this is correlated with rapid opening-up. While careful to indicate that this is all that their data prove, the British also suggested, even in the title to the cited paper, that the dermatan sulfate is released as part of fragments of the "dermatan sulfate proteoglycan" (decorin, Fig. 1) produced by proteolytic hydrolysis. Kronick,[4] on the other hand, found that decorin is still present in the alkaline protease-treated hide. His work thus does not support the interpretation of Alexander et al. However, Kronick's observation of the persistence of decorin was qualitative. He showed that (at least) a portion of the (core protein of) decorin in the hide survives alkaline protease treatment. But part of the proteoglycan may be removed. The British, on the other hand, showed that some dermatan sulfate was

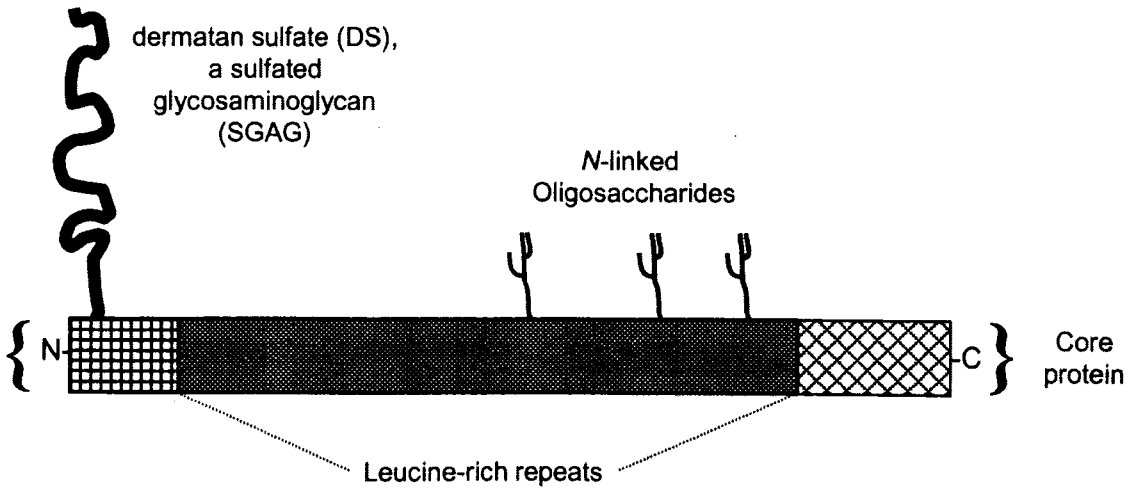

Figure 1. - A schematic representation of the decorin molecule. The core protein of bovine decorin has a molecular weight of ca. 43,000. The molecular weight of the dermatan sulfate, which is variable, is in the neighborhood of 50,000.

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emoved, but they also showed that some remains. To clarfy the situation, quantitative observations are needed. We herefore plan to measure both the decorin core protein conent and the sulfated glycosaminoglycan (SGAG) content of control hides and) hides that have been treated with alkaine protease or other proteases. The present work provides he method for measuring the SGAG content with a precision adequate for the indicated comparison.

Materials and Methods

Chemicals

(1) Alcian Blue 8GX, Fluka #05500, for microscopy, was purchased from Sigma-Aldrich Fine Chemicals, St. Louis, MO 63178.
(2) Dermatan sulfate (Chondroitin sulfate B), #C3788, was purchased from Sigma.
(3) Collagenase, from Clostridium histolyticum, #C9891, was purchased from Sigma.
(4) Protease inhibitor cocktail for mammalian tissues, #P8340, was purchased from Sigma.
(5) Guanidine hydrochloride, Mallinckrodt #7716, Bio-reagent grade, was purchased from Thomas Scientific, Swedesboro, NJ 08085.
(6) Triton X-100, electrophoresis purity reagent, #161-0407, was purchased from Bio-Rad Laboratories, Richmond, CA 94804.
(7) (Di)Methyl sulfoxide, reagent ACS grade, #41488, was purchased from Fisher Scientific, Pittsburgh, PA 15238.
(8) Sodium dodecyl sulfate, sequenal grade, #28364, was purchased from Pierce, Rockford, IL 61105.

Reagent solutions

(1) Acid-Detergent, 3X was 0.75% (w/v_t)* in Triton X-100, 54 mM in H_2SO_4.
(2) Acid-Detergent (0.25% (w/v_t)* in Triton X-100, 18 mM in H_2SO_4) was made by diluting Acid-Detergent,3X (#1 above) 1:3 (v/v_t)* with H_2O.
(3) Dye Solvent was 0.40M in guanidine hydrochloride, 18 mM in H_2SO_4.
(4) Alcian Blue, 25 g/l (nominal concentration) was prepared as follows: 0.625 g Alcian Blue 8GX was mixed thoroughly into 25 ml Dye Solvent (#3). The mixture was centrifuged at 12,000g for 30 minutes; the precipitate was discarded. The supernatant was stored in a dark bottle at 5°C for up to one month.
(5) Alcian Blue, 4g/l (nominal concentration) was prepared as follows: 1.60 ml supernatant (#4 above) was diluted with Dye Solvent to a volume of 10.0 ml.
(6) Alcian Blue Working Solution (0.2 g/l nominal concentration) was prepared as follows: A mixture of 5.0 ml Alcian Blue, 4g/l (#5 above), 1.00 ml 1.8M H_2SO_4, and 2.50 ml 10% (w/v_t)* Triton X-100 was diluted with water to a volume of 100 ml. The solution was stored in a dark bottle at 5°C for up to one week. To evaluate the concentration of Alcian Blue in the solution, 0.1 ml of the solution was diluted to 5 ml with Dye Solvent (#3 above), and the absorbance at 605 nm (A_{605}) was measured. A605 should be ca. 0.28.
(7) DMSO Wash Solution was 50 mM $MgCl_2$ in 40% (v/v_t)* dimethyl sulfoxide.
(8)TGS1 was 75 mM in tris(hydroxymethyl)aminomethane, 575 mM in glycine, 2.5% (w/v_t)* in sodium dodecyl sulfate.
(9)TGS2 was 75 mM in tris(hydroxymethyl)aminomethane, 575 mM in glycine, 0.5% (w/v_t)* in sodium dodecyl sulfate.
(10) Buffer: 10 mM $CaCl_2$, 100 mM tris(hydroxymethyl)-aminomethane (chloride), pH 6.80 ± 0.05 (20°C).
(11) Collagenase, 2500 U (vs collagen)/ml: Dissolve 25,000 units collagenase in 10 ml Buffer (#10).
(12) Dermatan Sulfate, 0.4 g/l in 4M guanidine hydrochloride.
*v_t is the final (total) volume.

Instruments

(1) Absorbance was measured with a model 14DS UV-Vis spectrophotometer made by Aviv Associates, Lakewood, NJ.
(2) Samples of bovine skin were milled under liquid nitrogen with a model 6800 CertiPrep freezer mill produced by Spex of Metuchen, NJ.
(3) Centrifugation was done in a Sorvall model RC-5B Refrigerated Centrifuge, Biomedical Division, Instrument Products, Du Pont Co., Newtown, CT 06470.

Assay procedure

(For a summary of the procedure, see Table I.)
After shaving the hair from a piece of fleshed hide, it is milled under liquid nitrogen in the Spex freezer mill. To a weighed 200-300 mg aliquot of the powdered hide (A1) are added 4 ml Buffer (Reagent Solution #10) and 1 ml Collagenase (#11). The mixture is incubated at 27°C for 10 minutes while shaking. After adding 5 ml 8M guanidine hydrochloride, the suspension is held at room temperature (24 ± 2°C) overnight. The remainder of the procedure is carried out at 5°C, except where otherwise stated, and centrifugation is at 12,000g (and 5°C) for 30-45 min, unless otherwise stated. Five ml of Acid-Detergent, 3X (#1) are added to the A1 suspension, and the suspension is incubated for 15 min; 75 ml Alcian Blue Working Solution (#6) are then added, and the mixture is held for 2 hours. The precipitate (A1') is collected by centrifugation, and the supernatant (A2') is discarded. To A1' are added 2.5 ml water, 2.5 ml 8M guanidine hydrochloride, and 2.5 ml Acid Detergent, 3X (#1). After mixing for 15 min at room temperature, the suspension is centrifuged at 12,000g and room temperature for

TABLE I

Summary of the Assay

Treatment #	Type	Fractionation: Discarded Fraction	Fractionation: Saved Fraction	Conditions: Guan.HCl (M)	Conditions: H_2SO_4 (mM)	Conditions: Triton % (v/vt)	Conditions: Alcian Blue (g/l)[2]	Other
			A1(Powdered Hide)					
1	Collagenase		↓					
2	Extraction		A1(Suspension)	4.0				
3	SGAG-AlBl[1] Ppt'n	A2'(Sup't)	A1'(Ppt)	0.44	18.0	0.25	0.2	
4	Dissolution of GAGs	B2(Ppt)	B1(Sup't)	2.67	18.0	0.25	0	
5	SGAG-A1B1 Ppt'n	C2(Sup't)	C1(Ppt)	0.44	18.0	0.25	0.2	DMSO-$MgCl_2$
6	Wash	D2(Sup't)	D1(Ppt)					Tris-Gly-SDS
7	Dissolution		D1(Solution)					
8	Measure A_{605}							

[1]A1B1= Alcian Blue [2]nominal concentration

30-45 min; the precipitate (B2) is discarded. To the supernatant (B1) are added, with stirring, 37.5 ml Alcian Blue Working Solution (#6). After incubating overnight, the precipitate (C1) is collected by centrifugation, and the supernatant (C2) discarded. C1 is washed by suspending it in 3.75 ml DMSO Wash Solution (#7), and mixing thoroughly for 15 min. After the suspension is centrifuged, the supernatant (D2) is discarded. The precipitate (D1) is suspended in 250 µl TGS1 (#8) and the mixture is stirred until the precipitate dissolves (ca.1 hour). Five ml TGS2 (#9) are then added, and the absorbance of the resulting solution (D1) at 605 nm is read against a suitable blank.

Dermatan Sulfate (#12) is run as a standard with every set of experiments.

Results and Discussion

Historical background

In our initial efforts to measure the SGAG content of bovine skin, we applied the procedure of Bjornsson[1] to skin samples extracted with guanidine hydrochloride. However, that procedure was designed for fluids such as synovial fluid and our results were discouraging, despite the care we took in preparing the extracts. After incorporating a suggestion by Bjornsson[5] (ref. 2, §3.4) to introduce an extra Alcian Blue precipitation step, and adopting some features of a more recent paper by Karlsson and Bjornsson on the quantitation of proteoglycans in biological fluids,[2] our results were somewhat improved, but still far from quantitative. We therefore examined the procedure step by step, and modified it as necessary.

The key reagent in the assay procedures cited above[1,2] is Alcian Blue 8GS.[6] Karlsson and Bjornsson[2] specify that the dye should be "certified for glycosaminoglycan/proteoglycan quantitation, Wieslab, Lund, Sweden". Alcian Blue 8GS is a variant of the commonly used Alcian Blue 8GX.[6,7] The two formulations differ only in the quantity and type of additives.[6] The 8GS formulation contains "much salt (NaCl and Na_2SO_4) and no stabilizer"; 8GX is "stabilized with boric acid/ sodium pentaborate" and contains little salt. The structure of the dye is shown in Fig. 2.[7,8] The dye was introduced into histochemistry for staining "mucins" by Steedman[9] a few years after its discovery in 1944,[8] and has been used for histochemical staining of "acid mucopolysaccharides" (acid glycosaminoglycans),[10,11] which includes SGAGs. John E. Scott, who contributed substantially to our

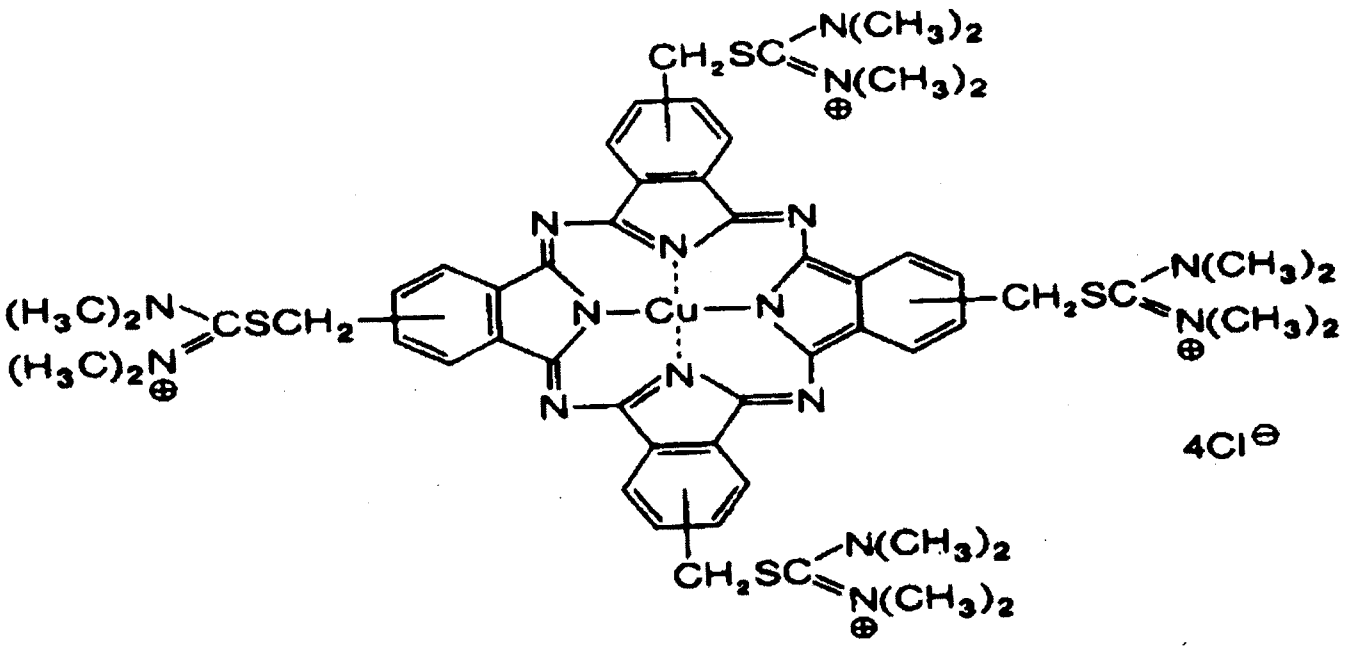

Figure 2. - The Structure of Alcian Blue 8GX. The commercial product was found to contain 2.9-3.0 S-methylene-tetramethyl-isothiouronium groups per molecule[6] (instead of 4 as shown).

resent understanding of the extracellular matrix[12] --- a natrix in whose structure decorin and other proteoglycans lay a predominant role --- reported in 1955 on the use of olutions of salts (e.g., KCl, $MgCl_2$, Na_2SO_4) "to dissolve recipitated [cetylpyridinium complexes of] carboxylic olysaccharides selectively, leaving untouched precipitates ontaining sulphated polysaccarides".[13] The concentration f salt at which the extent of dissolution of a given complex ncreases rapidly with increasing salt concentration was eferred to by Scott as the "critical salt concentration"[14] or later) as the "critical electrolyte concentration"[15] of the omplex. In the last-cited paper it was shown that various olyanions, including SGAGs, "took up the Alcian Blue dye vidly when the $MgCl_2$ concentration was 0.05-0.1M", and hat "sulfated tissues stained more intensely over a wider ange of $MgCl_2$ concentration (0.1-1.4M) than did the carboxylated and phosphorylated tissues (≤0.1M)". (They also noted that the critical electrolyte concentrations of glycosaminoglycans are better differentiated at pH 5.8 than under more acidic conditions.) The authors concluded that $MgCl_2$ permits "effective differentiation between polyanions containing sulfate groups and those containing carboxyl or phosphate groups".

Another criterion (in addition to critical electrolyte concentration) that permits differentiation of SGAGs from non-sulfated polyanions is pH. In 1956, Mowry reported that, at pH 2.7-3.0, connective tissue "mucins" stain bright blue with Alcian Blue 8GS, "with negligible staining of the background and nucleic acids".[16] Pearse stated that staining with Alcian Blue can be made specific for the sulfate group by "lowering the pH of the staining bath to something below 2" (ref. 10, p. 261).

Glycosaminoglycans (from urine) were quantitated, after separating them electrophoretically on cellulose acetate, by staining them with Alcian Blue, dissolving the complexes (blue spots), and measuring the absorbances of the resulting solutions.[17] This approach was used by Alexander et al. in their study[3] of "proteoglycan removal" from hides in the presence and absence of an alkaline protease. Bjornsson and Karlsson[1,2] refined the quantitation of SGAGs considerably. They used a pH of 1.5 to convert the carboxyl groups of proteins and non-sulfated glycans such as hyaluronan into their uncharged, protonated, form, while maintaining the ester sulfate groups of the SGAGs unprotonated and charged, and applied the principle of critical electrolyte concentration to alternately and selectively dissolve and precipitate the complexes of the SGAGs with Alcian Blue. They were thereby able to separate the SGAGs from non-sulfated GAGs (glycosaminoglycans) by purely chemical means, i.e., without resorting to any physicochemical procedure such as electrophoresis. As the electrolyte they used guanidinium chloride (rather than an inorganic salt such as $MgCl_2$), 2.67-4.0M to dissolve Alcian Blue-SGAG complexes (together with any non-sulfated GAGs), and 0.40-0.45M to precipitate the Alcian Blue-SGAG complexes (leaving non-sulfated GAGs in solution). The use of guanidinium chloride is particularly attractive for analysis of solid tissues such as

most efficient agent for the extraction of proteoglycans, including those containing SGAGs (ref. 18, pp. 779-780). Hydrophobic interaction of proteins with the dye is prevented by the use of the detergent Triton X-100[1].

EXPERIMENTAL

When we first tried Bjornsson's recent method, it appeared to be good for detection, but not for quantitation. All of the samples containing SGAG (dermatan sulfate) yielded about the same color (absorbance), regardless of the amount of SGAG present. This could be attributed to either (1) an inadequate amount of the dye, Alcian Blue, (2) too much SGAG, or (3) our use of Alcian Blue 8GX instead of the variety specified by Karlsson and Bjornsson.[2] We found that the first two explanations were both correct. When the dye (8GX) concentration was increased --- by a factor of more than 3 --- to the level used in our current procedure (Materials and Methods), and the amount of SGAG was decreased to the level used in our current procedure, a standard curve linear to high absorbance values was obtained (Fig. 3). It therefore became unnecessary to further consider the third possibility. The slope of the line shown in Fig. 3 gives a color yield (A_{605}) per unit SGAG of 9.87±0.74 AU/cm per mg DS (dermatan sulfate).

The above experiment (Fig. 3) was run as described in the

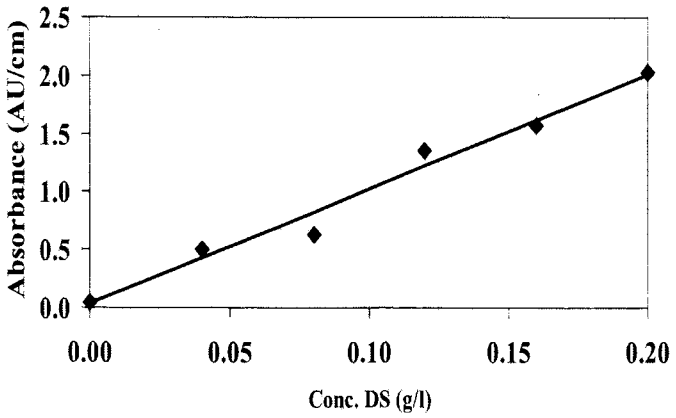

Figure 3. - Dependence of Color Yield (A_{605}) on the concentration of SGAG in the sample

section on "Assay Procedure" (Materials and Methods) except as follows: (1) The samples were 1 ml aliquots of 6 solutions of dermatan sulfate (DS) in 4M guanidine hydrochloride varying in concentration from 0 to 0.2 g DS/l. (2) Additions of Buffer (#10) and Collagenase (#11) were omitted. (3) In the initial part of the procedure, the volumes at every stage were 1/10th of the volumes specified in the "Assay Procedure". Thus, 0.5 ml Acid-Detergent, 3X (#1), and 7.5 ml Alcian Blue Working Solution (#6) were added

cedure, the volumes at every stage were scaled down by a factor of 5 from the volumes specified in the "Assay Procedure". Thus, 0.5 ml each of water, 8M guanidine hydrochloride, and Acid-Detergent 3X (#1) were added to A1', and 7.5 ml Alcian Blue Working Solution (#6) to B1. The remainder of the procedure was as described in Materials and Methods. It should be noted that, despite the above variations, the conditions given in Table I were adhered to during every step of the experimental procedure.

To obtain the color yielded by samples of hide, the "Assay Procedure" (Materials and Methods) was run on 7 samples of powdered hide, except as follows: (1) The sample size varied from 0 to 300 mg. (2) Additions of Buffer (#10) and Collagenase (#11) were omitted. (3) Ten ml of 4M guanidine hydrochloride were added to the sample of powdered hide. The conditions given in Table I held throughout. We again obtained results that were linear to high absorbance values (Fig. 4). On the basis of these results we decided to use 200-300 mg samples of powdered hide for routine analysis of SGAG content.

The slope of the line in Fig. 4 gave a color yield per unit

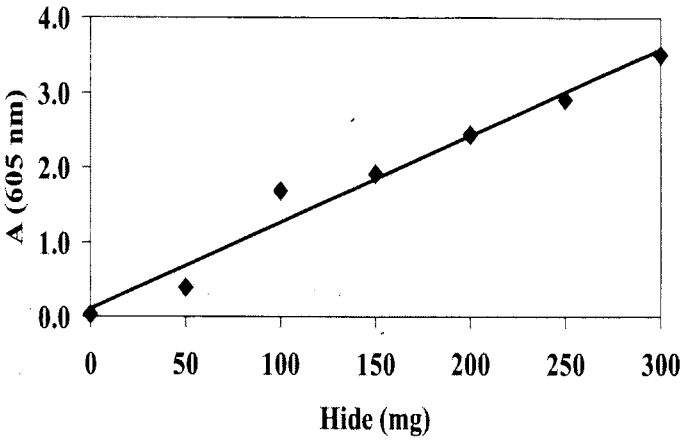

Figure 4. - Dependence of Color Yield (A_{605}) on Skin Sample Weight

weight of powdered hide of 11.5±0.9 AU/cm per g hide. Dividing this by the color yield per unit weight of DS (9.87±0.74 AU/cm per mg DS), one obtains as the SGAG content of bovine hide 1.17±0.13 mg SGAG/g hide. In calculating the standard deviation (0.13) of the quotient, we applied the equation[19]

$$(\sigma_u/u)^2 = (\sigma_v/v)^2 + (\sigma_w/w)^2 \qquad (1)$$

where u = v/w, v = the color yield per g hide, w = the color yield per mg DS, and σ = standard deviation.

In the course of the assay, SGAG-Alcian Blue precipitates are collected by centrifugation. A potentially serious problem with some of the precipitates, especially those collect-

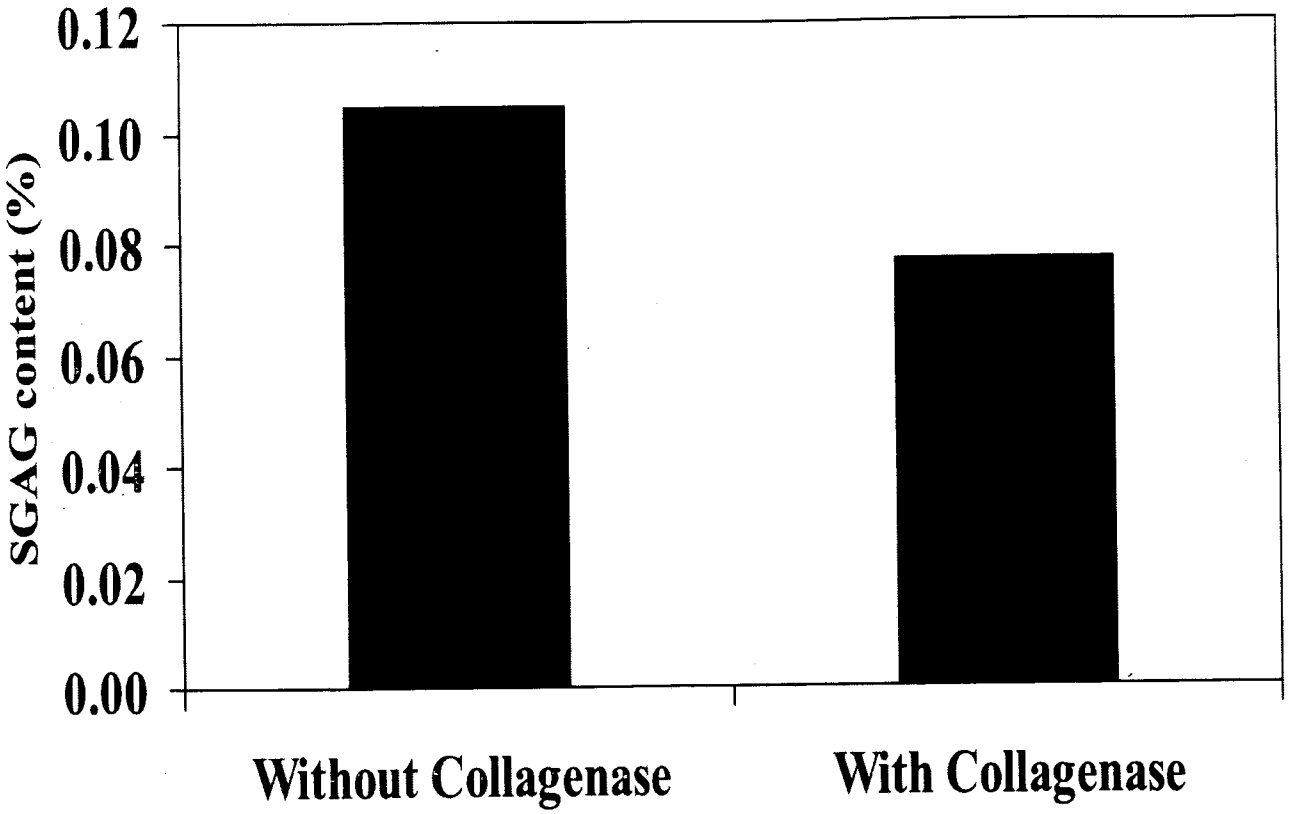

Figure 5. - The Effect of Prolonged Exposure of Hide to Collagenase on the Measured Color Yield (A_{605}).

ed early in the assay procedure, was that they floated, instead of adhering to the inner surface of the centrifuge tube. There was therefore a high risk of losing some of the precipitate while separating it from the liquid around it. Reasoning that the problem was probably related to the fact that the liquid is slimy and viscous, and recognizing that the viscosity is mainly due to the presence of a great deal of collagen, we treated the solutions with collagenase. That resolved the floating problem; all precipitates collected after collagenase treatment adhered to the centrifuge tube. However, as shown in Fig. 5, the treatment with collagenase reduced the yield of SGAG by about 25%. This may be related to the tight association of decorin --- the predominant proteoglycan in skin --- with collagen. Collagen is known to protect decorin from proteases; perhaps it also protects decorin from chondroitinases. Whatever the explanation, we decided to reduce the exposure to collagenase. We had used a substantial amount of the enzyme and had incubated for 4 hours. We found that the incubation time could be reduced to 10 minutes without losing the benefit of the enzyme treatment.

To determine the SGAG content of our preparation of powdered hide, we conducted a more extensive experiment than that previously described using 15 samples of the powdered hide and 15 samples of the DS solution (#12). These were treated as described in "Assay Procedure" (M & M) except as follows: (1) The hide samples varied in weight from 200.0 to 208.1 mg. (2) To each of the hide samples we added 4.75 ml Buffer (#10) and 250 µl Collagenase (#11). (3) Each sample of DS consisted of 5 ml of "Reagent Solution" #12. (4) No buffer or collagenase was added to the DS samples. (5) Five ml 4M guanidine hydrochloride were added to each sample of DS. This yielded a solution corresponding to A1(Suspension) (Flowchart, Table 1, step 2). The data yielded a value of 1.14±0.17 mg SGAG/g powdered hide as the SGAG content of our preparation of powdered hide, which agrees very well with our previous results. Again, the standard deviation includes error in both the DS set of runs and the powdered hide set of runs.

CONCLUSIONS

A procedure suitable for the comparison of the SGAG content of bovine hides subjected to different treatments is described in the Materials and Methods section and summarized in Table I. We intend to apply the procedure to determine the efficacy of various proteases, including a halophile protease which is active in the presence of concentrated salt, in removing SGAGs from the hide. To avoid secondary effects such as changes in water content on SGAG content, the latter will be related to the collagen content of the hide samples, as was done, for example, by Alexander et al.[3]

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