Preface

Proteoglycans are some of the most elaborate macromolecules of mammalian and lower organisms. The covalent attachment of at least five types of glycosaminoglycan side chains to more than forty individual protein cores makes these molecules quite complex and endows them with a multitude of biological functions. *Proteoglycan Protocols* offers a comprehensive and up-to-date collection of preparative and analytical methods for the in-depth analysis of proteoglycans. Featuring step-by-step detailed protocols, this book will enable both novice and experienced researchers to isolate intact proteoglycans from tissues and cultured cells, to establish the composition of their carbohydrate moieties, to generate strategies for prokaryotic and eukaryotic expression, to utilize methods for the suppression of specific proteoglycan gene expression and for the detection of mutant cells and degradation products, and to study specific interactions between proteoglycans and extracellular matrix proteins as well as growth factors and their receptors. The readers will find concise, yet comprehensive techniques carefully drafted by leading experts in the field.

Each chapter commences with a general Introduction, followed by a detailed Materials section, and an easy-to-follow Methods section. An asset of each chapter is the extensive notation that includes troubleshooting tips and practical considerations that are often lacking in formal methodology papers. The reader will find this section most valuable because it is clearly provided by experienced scientists who have first-hand knowledge of the techniques they outline. In addition, most of the chapters are well illustrated with examples of typical data generated with each method. I have made a special effort to ensure a detailed, step-by-step rendition of the methods that would help in reproducing each experimental protocol.

Proteoglycan Protocols is divided into three sections. Part I focuses on the general protocols dedicated to the isolation and purification of proteoglycans from tissues and cells of vertebrates and invertebrates. In nineteen chapters, the most commonly used protocols are reviewed by expert scientists. The reader will be able to isolate proteoglycans from specialized tissues, cell culture, body fluids, analyze the glycosaminoglycan side chains and the protein core, and investigate intracellular biosynthetic events using both qualitative and quantitative approaches.

Part II focuses on the expression, detection, and degradation of proteoglycans. Twenty chapters cover most of the current knowledge regarding these issues of proteoglycan biology. The reader will find protocols describing various approaches in recombinant gene expression systems, together with theoretical and practical gene targeting approaches, using both antisense technology and somatic cell targeting. A variety of unique approaches will enable the reader to inhibit glycosaminoglycan

synthesis, to identify cell mutants in proteoglycan biosynthesis, and to study the degradation of various glycosaminoglycans by using chemical and enzymatic methods. In addition, strategies are described for the identifications in adult human tissues and fluids of proteoglycan degradation products.

Part III comprises twelve chapters focused on understanding the complex interactions between proteoglycans—either the protein or the glycosaminoglycan moiety—and various extracellular matrix proteins, growth factors, and receptors. This is a blooming area that will undoubtedly expand in the near future as we enter the era of proteomics. The reader will find protocols devoted to the theoretical and practical understanding of specific proteoglycan interactions using affinity-based approaches, including affinity coelectrophoresis, affinity chromatography, and optical biosensor and phage display technologies. In addition, proteoglycan interactions with receptor tyrosine kinases and lipoproteins are also covered.

Proteoglycan Protocols has been developed through the efforts of ninety-seven scientists representing ten countries and three continents. I wish to thank all of the authors for their contributions and their outstanding job in summarizing complex protocols and for providing extensive notations. The latter, I believe, will make a crucial difference in the success rate of each experimental strategy.

I would like to thank the Series Editor, John Walker, and the production team at Humana Press for their contributions to the successful realization of this multiauthor book. I am particularly indebted to my assistant Kit Foster for her dedication and continuous effort in this endeavor. She played a key role in the assembly, editing, formatting, general organization, and index preparation of this volume.

From my personal vantage point, I learned immensely during the process of assembling and editing the chapters. I am thankful for that and amply rewarded.

Renato V. Iozzo, MD

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Isolation of Proteoglycans from Tendon

Kathryn G. Vogel and Julie A. Peters

1. Introduction

Proteoglycans make up less than 1% of the dry weight of a dense connective tissue such as tendon ***(1)***. Most of this proteoglycan is a small molecule called decorin. Because decorin has only one glycosaminoglycan chain, it cannot be separated from other proteins by the CsCl density-gradient centrifugation method that was originally used to purify aggrecan from cartilage.

The basic approach described in this chapter is to extract proteoglycans from the tissue with 4 *M* guanidine, a solution that will denature collagen and disrupt most kinds of noncovalent molecular interactions. The cross-linked collagen of adult tendon remains insoluble during this extraction, allowing the proteoglycans and other soluble proteins to be separated from the bulk of the tissue by centrifugation. Proteoglycans are then separated from other extracted proteins by ion-exchange chromatography, taking advantage of the anionic nature of the glycosaminoglycan chain.

This method has been used to quantitate and isolate proteoglycans from the tensile (proximal) and compressed (distal) regions of bovine deep flexor tendon. These mechanically distinct regions of flexor tendon are characterized by differences in proteoglycan amount and type ***(2)***. The method is equally applicable to isolation of proteoglycans from human tendon or from other dense connective tissues ***(3)***. Once isolated, the large proteoglycans can be separated from smaller ones by sieve chromatography. These isolated proteoglycans and their unique core proteins and glycosaminoglycan chains are of sufficient purity to then be examined by specific analytical techniques or used in functional assays.

2. Materials

2.1. Quantitation of Glycosaminoglycans

1. Single-edge razor blades.
2. Heated water bath.

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3. Microcentrifuge.
4. Lyophilizer.
5. Papain digestion solution: 0.05 *M* sodium acetate, 5 m*M* L-cysteine HCl, 10 m*M* EDTA, pH 5.5. Add 50 µg/mL papain just before starting digestion.
6. DEAE cellulose (Whatman, DE52).
7. Disposable columns (Bio-Rad Poly Prep, 0.8 × 4 cm).
8. Column elution solutions: 0.02 *M* HCl, 1 *M* HCl.
9. Uronic acid detection reagent (orcinol reagent): 2 g of orcinol (5-methylresorcinol; 3,5-dihydroxytoluene monohydrate) in 50 mL of 1.5% $FeCl_3$, 850 mL of conc. HCl, and 150 mL of water ***(4)***. Orcinol is sensitive to light and is irritating to eyes, the respiratory system, and skin. Dissolve orcinol in $FeCl_3$ solution, then add HCl and water. Use caution and work in a fume hood when adding water to concentrated HCl.
10. Uronic acid standards: stock solution of 100 µg/mL of glucuronolactone diluted to standards of 5 µg/mL, 10 µg/mL, 20 µg/mL, and 30 µg/mL.
11. Pyrex test tubes.
12. Heat block.
13. Spectrophotometer.

2.2. Isolation of Proteoglycans

1. Single-edge razor blades.
2. Tissue-grinding device (we have used a food mill made by Arthur Thomas, Incorporated, Philadelphia, PA).
3. Lyophilizer.
4. 16-mL centrifuge tubes with sealing screw cap (Nalge Nunc).
5. 4 *M* guanidine buffer: 4 *M* guanidine, 0.05 *M* sodium acetate, pH 6.0. Add protease inhibitors just before starting extraction: 5 m*M* benzamindine, 5 m*M* phenylmethylsulfonyl fluoride, and 1 m*M* N-ethylmaleimide.
6. Nutator (Clay-Adams) or other rocking device.
7. Centrifuge (Beckman J2, JA-17 rotor).
8. 7 *M* urea buffer: 7 *M* urea, 0.05 *M* sodium acetate, pH 6.0.
9. 7 *M* urea buffer plus protease inhibitors: shortly before use add 5 m*M* benzamindine, 5 m*M* phenylmethylsulfonyl fluoride, 1 m*M* N-ethylmaleimide.
10. Dialysis tubing (MWCO: 12,000–14,000).
11. DEAE cellulose (Whatman, DE52).
12. Disposable columns, Bio-Rad Poly-Prep, 0.8 × 4 cm.
13. Column elution solutions: 7 *M* urea buffer + 0.1 *M* NaCl; 7 *M* urea buffer + 0.2 *M* NaCl; 7 *M* urea buffer + 0.8 *M* NaCl.

2.3. SDS Polyacrylamide Gel Electrophoresis of Proteoglycans

1. 2-mL screw-capped centrifuge tubes.
2. 95% ethanol.
3. Microcentrifuge.
4. Enzyme digestion buffer: 0.1 *M* Tris base, brought to pH 8.0 with acetic acid.
5. Chondroitinase ABC (Seikagaku, 5 U/vial) resuspended in 0.5 mL of 0.1 *M* Tris-HCl, pH 8.0 mL (10 U/mL).
6. Gel sample buffer: for 100-mL, combine 50 mL stacking gel buffer, 5 g sodium dodecyl sulfate, 20 g glycerol, 4 mg bromophenol blue, and water to volume.
7. Electrophoresis apparatus and power source.

Table 1
Water Content of Adult Flexor Tendons

Tissue	1	2	3	
Tens.	65.6	61.5	62.7	
	65.2	62.1	63.4	$\bar{X}$ = 63.7 + 1.7 %
	66.6	62.1	63.7	
Comp.	70.2	70.6	73.1	
	70.8	67.3	71.1	$\bar{X}$ = 71.0 + 1.7 %
	70.9	70.9	73.7	

Samples of fresh tendon from animals between 1 and 2 years of age were cut into small pieces with a razor blade, weighed, lyophilized, and weighed again to determine the percent of tendon wet weight that was water. Triplicate samples of about 130 mg each were assessed. *Tens.*, tissue from the tensile/proximal region of tendon; *Comp.*, tissue from the compressed/distal region of tendon. Samples are from the front (1) and rear (2) leg of one animal and the rear (3) leg of another. Note that the water content is lower in the tensile tendon.

8. Polyacrylamide: 30% acrylamide, 0.8% bis-acrylamide.
9. Separating gel buffer: 0.75 *M* Tris, 4 m*M* EDTA, pH 8.8.
10. Stacking gel buffer: 0.25 *M* Tris, 4 m*M* EDTA, pH 6.8.
11. 0.5% ammonium persulfate.
12. TEMED.
13. Electrode buffer stock: 0.1 *M* Tris, 0.768 *M* glycine, 8 m*M* EDTA, pH 8.7. After adjusting pH (if necessary), add 0.4% SDS. The upper buffer is diluted 1/4; the lower buffer is diluted 1/8.
14. Gel fixative: 50% methanol, 7% acetic acid, 43% dH_2O.
15. Coomassie blue staining solution: 0.1% Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid, 50% dH_2O.
16. Gel destain: 25% methanol, 7% acetic acid, 68% dH_2O.
17. Alcian blue staining solution: 0.5% Alcian blue (8GX, Sigma Chemical Co), 7% acetic acid, 93% dH_2O.
18. Alcian blue destain solution: 7% acetic acid, 93% dH_2O.

3. Methods

3.1. Quantitation of Glycosaminoglycans

1. Dissect tendon tissue free of muscle and fat, cut into chunks about 3 mm on a side, weigh, lyophilize, weigh again (*see* **Note 1** and bovine tendon water content, **Table 1**).
2. Add papain digestion solution to tissue at 50 mg dry tissue/1 mL enzyme in 2-mL screw cap tubes. Incubate at 65°C until tissue pieces have disappeared (6–18 h, *see* **Note 2**).
3. Centrifuge and remove the supernatant. Dilute supernatant with equal volume of dH_2O to assure low salt concentration.
4. Pour columns containing 0.3 mL DE-52 in water (*see* **Note 3**).
5. Apply 1 mL of diluted sample to DEAE cellulose in column; collect the flow-through (*see* **Note 4**).
6. Rinse column with 20 mL of 0.02 *M* HCl to remove loosely adherent molecules. Elute the glycosaminoglycans into a clean tube with 2 mL of 1 *M* HCl.

Table 2
Uronic Acid Content of Adult Flexor Tendons (μg uronic acid/mg dry weight)

Tissue	1	2	3	
Tens.	3.1	2.6	2.1	
	2.8	2.2	2.3	$\bar{X} = 2.6 \pm 0.4$
	3.3	2.2	2.4	
Comp.	7.1	7.1	6.0	
	5.8	6.8	6.3	$\bar{X} = 6.4 \pm 0.8$
	5.2	7.9	5.7	

Samples of dry tendon were digested with papain, passed over a small column of DEAE cellulose, and the isolated glycosaminoglycans assessed for uronic acid content. Note that tissue from the compressed region of tendon contains a higher amount of uronic acid.

7. Determine the amount of GAG uronic acid spectrophotometrically using the orcinol reagent and uronic acid standards ***(4)***. Combine 0.5 mL of glycosaminoglycan sample or standard with 1.5 mL of orcinol reagent in Pyrex test tube, place in heat block at 100°C for 20 min, cool to room temperature, read OD at 670 nm within 30 min, and calculate micrograms of uronic acid per milligram of tissue dry weight (*see* **Notes 5–7**, and tendon uronic acid content, **Table 2**)

3.2. Isolation of Proteoglycans

1. Chop the tissue into chunks ~3 mm on a side and freeze immediately with liquid nitrogen (*see* **Note 8**).
2. Powder the frozen chunks of tissue (*see* **Note 9**).
3. Extract tissue in 4 *M* guanidine buffer + protease inhibitors for 24 h at 4°C with rocking. A good ratio of tissue wet weight to extraction fluid is about 1/20 (*see* **Note 10**). The efficiency of proteoglycan extraction from powdered tendon was higher than extraction from tissue chunks (*see* **Tables 3** and **4**).
4. Centrifuge in Beckman JA-17 fixed-angle rotor at 3440*g* (5000 rpm) for 10 min; carefully remove the supernatant.
5. Repeat the extraction with fresh buffer, centrifuge, and combine supernatants (*see* **Note 11**).
6. Dialyze the extract into 7 *M* urea buffer + 0.1 *M* NaCl (*see* **Notes 12** and **13**).
7. Apply 0.5 mL of dialyzed extract to a 1-mL column of DEAE cellulose equilibrated in 7 *M* urea buffer (*see* **Note 14**).
8. Rinse the column with 8 mL of 7 *M* urea buffer + 0.1 *M* NaCl followed by 3 mL of 7 *M* urea buffer + 0.2 *M* NaCl.
9. Elute proteoglycans with 1.5 mL of 7 *M* urea buffer + 0.8 *M* NaCl (*see* **Note 15**).

3.3. SDS Polyacrylamide Gel Electrophoresis of Proteoglycans

1. Precipitate the proteoglycans in ethanol to prepare samples for gel electrophoresis. For example, mix 50 μL of sample from the ion-exchange column with 400 μL of ethanol in a small centrifuge tube. Let stand at –20°C for several hours or in –70°C freezer for at least 1h.

Table 3
Extraction of Proteoglycan from Adult Flexor Tendons

	Tensile		Compressed	
	Chunks	Powder	Chunks	Powder
7 M Urea				
	1. 47	41	63	131
	2. 17	35	28	41
Total	64	76	91	172
7 M Urea + *0.1 M NaCl*				
	1. 141	195	285	624
	2. 79	110	131	122
Total	220	305	416	746
4 M Guanidine				
	1. 137	176[a]	748	1335
	2. 83	330	239	112
Total	220	506	987	1447

[a]Due to tissue swelling, 15 mL of solution was added.

Chunks or powdered tendon was extracted with 7 *M* urea, 7 *M* urea + 0.1 *M* NaCl, or 4 *M* guanidine. In each case the extraction started with 200 mg dry weight of tissue and 8 mL of extraction solution. After 24 h the extracts were centrifuged, the supernatants removed, and an additional 8 mL of extraction solution added to the pellet for another 24 h. The amount of uronic acid in the supernatant of each extraction is shown, in micrograms. 1, first extraction; 2, second extraction of same tissue.

2. Spin samples in the microcentrifuge in cold for 10 min at 10,000 rpm, remove supernatant by suction, rinse pellet and tube with 1 mL cold ethanol, let stand at –20°C for at least 1h, and spin again. Dry pellet with brief lyophilization (*see* **Note 16**).
3. Solubilize pellet in 25 µL of gel sample buffer and heat to 100°C for 5 min in a heat block. If the samples are to be reduced, add dithiothreitol or β-mercaptoethanol to the gel sample buffer before heating.
4. The core protein of proteoglycans having chondroitin sulfate or dermatan sulfate glycosaminoglycan chains can be seen after removal of the glycosaminoglycans by digestion with chondroitinase ABC (*see* **Note 17**). Resuspend the dry proteoglycan pellet in 25 µL of digestion buffer, add 1 µL (0.01 unit) of enzyme, and incubate at 37°C for 1 h. Add 25 µL of 2X gel sample buffer and heat to 100°C for 5 min in the heat block.
5. Pour 4–16% gradient gel, 1.5 mm thick (***5**, see* **Notes 18** and **19**). For one gel, make solutions containing 4% and 16% acrylamide. For 4%: 6.75 mL separating buffer, 1.8 mL acrylamide + bis, 4.14 mL water, 135 µL 10% SDS, 13.5 µL TEMED, and 675 µL 0.5% ammonium persulfate. For *16%:* 3.38 µL 2X separating buffer, 7.2 mL acrylamide + bis, 2.12 mL water, 135 µL 10% SDS, 13.5 µL TEMED, and 675 µL 0.5% ammonium persulfate. The 16% solution is stirred while pumping it between glass plates, as it is diluted by the 4% solution.
6. Pour 4% stacking gel using a 15-lane comb.

Table 4
Efficiency of Proteoglycan Extraction[a]

	Tensile	Compressed
7 *M* urea	16%	12%
7 *M* urea + 0.1 *M* NaCl	65%	51%
4 *M* Guanidine	108%	100%

[a] Uronic acid in the papain digest of each tissue was set to equal 100% tens. = 467 μg, comp. = 1454 μg.

The amount of uronic acid solublized by two sequential 24-h extractions in each extraction solution (**Table 3**) is compared to the amount of uronic acid measured after papain digestion of tissue from the same animal (sample 2, **Table 2**). Note that adding 0.1 *M* NaCl to 7 *M* urea greatly increased the amount of proteoglycan that was solublized from the tissue. Virtually all proteoglycan was solublized by 4 *M* guanidine.

7. Load 10– 50 μL of sample into each lane, as appropriate. Put molecular weight standards in one lane. If samples were digested with chondroitinase ABC, it is useful to prepare one sample containing only 1 μL of enzyme and digestion buffer.
8. Run electrophoresis at 8 mA for approximately 18 h (*see* **Note 20**). Cooling to 16°C may help uniformity but is not necessary. If desired, carry out Western blot procedures immediately after electrophoresis.
9. Put the gel into gel fixative solution for 1 h.
10. Cover the gel with Coomassie blue staining solution for 1h with gentle rocking. Pour off the stain. Cover the gel with several changes of destain solution until the gel background is clear.
11. To stain for proteoglycans, cover the gel in Alcian blue staining solution for 1 h. Pour off the stain and destain with 7% acetic acid until gel background is clear (*see* **Fig. 1** and **Notes 21** and **22**).

4. Notes

1. Many plastic tubes will lose weight during lyophilization. It is best to remove dry tissue from the tube to obtain dry weight.
2. Tissue digestion can be encouraged by shaking the tube and by adding additional papain. The digest may appear cloudy.
3. Precycle DE52 through washes in 0.5 *N* HCl, dH_2O, 0.5 *N* NaOH, and then rinse in dH_2O until filtrate is near pH 7. Used resin can be recovered by the same treatment and used again.
4. The DE52 resin is kind; the columns will not run dry even when no fluid remains above the resin. Columns can be placed in a tube to collect eluent and simply moved to the next tube for the next elution fluid.
5. Place a glass marble on each tube while in the heat block to diminish evaporation of acid. Cool tubes quickly and uniformly by transferring the tubes to a rack and placing the rack in a pan of water.
6. Dilute the sample with dH_20 as needed (1/2 or 1/5) to obtain readings that fall within the range of standards. Authentic chondroitin sulfate was recovered from the columns with efficiency >95%. Multiply by 3.2 to convert amount of uronic acid to chondroitin sulfate.
7. Orcinol reagent is stable for 6 wk when kept in the refrigerator in a dark glass container.

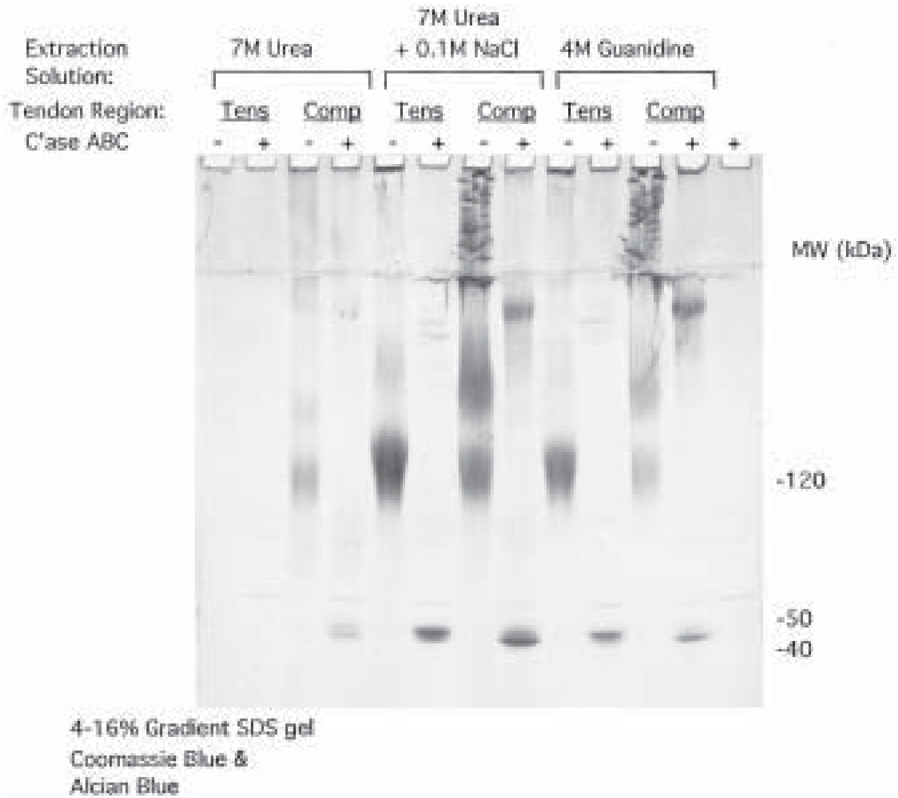

Fig. 1. SDS/Polyacrylamide gel electrophoresis of proteoglycans. Proteoglycans were extracted, isolated by ion-exchange chromatography, precipitated with ethanol, incubated without or with added chondroitinase ABC, and loaded onto a 4–16% gradient gel. After electrophoresis the gel was stained with Coomassie blue and Alcian blue. An approximately equal amount of uronic acid (12.5 μg) from samples extracted with 7 *M* urea + 0.1 *M* NaCl and 4 *M* guanidine was loaded onto each lane; it was not possible to precipitate this much material from the 7 *M* urea extracts. *Tens,* from tensile region of tendon; *Comp,* from compressed region of tendon. Note that a proteoglycan migrating just above 120 kDa (decorin) is the predominant molecule in tensile samples. A proteoglycan that enters the running gel but migrates more slowly (biglycan) is present only in the compressed samples. The core proteins of these small proteoglycans migrate close together at about 45 kDa. Large proteoglycan (aggrecan) is present in the stacking gel of compressed samples but does not form a discrete band. The high-molecular-weight band appearing after chondroitinase ABC digestion of samples from compressed tissue is the aggrecan core protein with some keratan sulfate chains.

8. To freeze tissue chunks for storage, put a few at a time into a half-liter plastic container, add a small amount of liquid nitrogen, cover loosely, and shake the container. This makes it possible subsequently to remove individual chunks of frozen tissue. Be sure to maintain a vent to allow gas to escape during freezing.

9. Tendon is difficult to powder. To avoid gumming up the mill, it is necessary to keep the grinding surfaces cold by adding liquid nitrogen continually. Some loss of tissue is inevitable. For smaller amounts of tissue one can use a tissue macerator (a shaking steel ball and chamber) cooled with liquid nitrogen.
10. The ratio of extraction fluid to tissue can be varied, depending on the goal of the extraction. Tensile tendon swells a great deal during extraction, whereas compressed tendon and cartilage do not. Powdered tendon swells more than chunks of tissue. For highest extraction efficiency it is important to have a large supernatant volume and a small pellet after centrifugation. However, a large supernatant volume is not desirable during the subsequent dialysis.
11. With sufficient fluid volume, virtually 100% of the proteoglycan can be removed from powdered tendon with two sequential extractions in 4 *M* guanidine buffer (*see* **Tables 3** and **4**). In contrast, extraction with 7 *M* urea solublized less that 20% of the proteoglycan. Addition of 0.1 *M* NaCl to 7 *M* urea increased extraction efficiency fourfold compared to extraction in 7 *M* urea alone, but still solublized only 50–65% of the total proteoglycan (*see* **Table 4**). **Figure 1** indicates that these two extraction solutions solublize the same proteoglycans.
12. It is necessary to remove 4 *M* guanidine in order to carry out ion-exchange chromatography. Efficient dialysis is accomplished during three sequential 24-h dialysis steps using 5 volumes of 7 *M* urea buffer for each step. This will reduce guanidine concentration to less than 0.04 *M*, a level that does not impede glycosaminoglycan binding to the anion-exchange resin.
13. Although extraction in 7 *M* urea + 0.1 *M* NaCl is less efficient than extraction in 4 *M* guanidine, it eliminates the need to dialyze samples before ion-exchange chromatography.
14. With a larger extract volume, one can use a larger DEAE cellulose column. The extract can be pumped onto the column and eluted with a continuous gradient of NaCl from 0.1 to 0.8 *M*. Proteoglycans will elute at about 0.25 *M* NaCl ***(6)***.
15. The yield of decorin after ion-exchange chromatography and sieve chromatography was about 150 μg/g wet weight of adult tensile bovine tendon ***(6)***.
16. Precipitate proteoglycans in 8 volumes of ethanol. Precipitation from 7 *M* urea + NaCl does not present a problem. However, it is sometimes useful to precipitate the 4 *M* guanidine extract. In this case it is important to remove all supernatant and rinse the tube carefully. If any guanidine remains in the sample, it will form a nasty precipitate with SDS in the gel sample buffer and make electrophoresis impossible.
17. The resuspended chondroitinase ABC enzyme from Seikagaku can be kept in the refrigerator for a year.
18. All electrophoresis buffers should be made with Tris base and brought to proper pH with HCl.
19. It is not necessary to run gradient gels to see proteoglycan. However, the 4–16% gel is useful for visualizing intact biglycan and decorin and their core proteins on the same gel.
20. Large proteoglycans such as aggrecan will not enter the separating gel. As the gel is running, it is possible to see "crinkly" diffraction lines in the stacking gel of lanes containing the large proteoglycan.
21. The various concentrations of methanol suggested for destaining solutions are designed to reduce methanol consumption. After destaining in 7% acetic acid, the gel will be somewhat swollen; it will return to size when stored in the solution containing 25% methanol. If the protein bands have faded, just add a few drops of Coomassie blue to this final solution.
22. Gel electrophoresis can be used to visualize intact proteoglycans in a tissue extract, without ion-exchange purification, by staining only with Alcian blue. The gel should be washed

with at least three changes of destain solution over a 24-h period to assure complete removal of SDS; then stain the gel with Alcian blue and destain in 7% acetic acid. If SDS remains in the gel, it will bind Alcian blue and make the gel impossible to destain. Do not stain samples of the total extract with Coomassie blue, because this produces a blue smear that will obscure the proteoglycans.

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References

1. Vogel, K. G. and Heinegard, D. (1985) Characterization of proteoglycans from adult bovine tendon. *J. Biol. Chem.* **260,** 9298–9306.
2. Vogel, K. G. (1995) Fibrocartilage in tendon: a response to compressive load, in *Repetitive Motion Disorders of the Upper Extremity* (Gordon, S. L., Blair, S. J., and Fine, L. J., eds.) American Academy Orthopedic Surgery, Rosemont, IL, pp. 205–215.
3. Berenson, M. C., Blevins, F. T., Plaas, A. H. K., and Vogel, K. G. (1996) Proteoglycans of human rotator cuff tendons. *J. Orthoped. Res.* **14,** 518–525.
4. Brown, A. H. (1946) Determination of pentose in the presence of large quantities of glucose. *Arch. Biochem.* **11,** 269–278.
5. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
6. Vogel, K. G., Sandy, J. D., Pogany, G., and Robbins, J. R. (1994) Aggrecan in bovine tendon. *Matrix Biol.* **14,** 171–179.