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Rapid size-exclusion chromatography of proteoglycans

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Abstract

The separation of intact proteoglycans using high-performance liquid chromatography is not trivial because the high molarity denaturing buffers required to maintain proteoglycans in the disaggregated state create back-pressures higher than the limits of many HPLC systems. Until recently, low back-pressure requirements of HPLC size-exclusion columns precluded their use for the separation of intact proteoglycans. In this study we show that rapid size-exclusion chromatography is possible in 8 *M* urea buffers using a Dionex BioLC system equipped with a Bio-Rad BioSil Sec-400 column. This technique reduced the time required for size-exclusion chromatography of intact proteoglycans from approximately 18 h (Sepharose CL4B) to 25 min and in some cases improved resolution of the sample.

Keywords: Proteoglycans

1. Introduction

Proteoglycans consist of protein cores substituted with highly sulfated glycosaminoglycan (GAG) chains and these molecules are thus anionic in nature. This anionic character is partially responsible for the binding of proteoglycans to many extracellular matrix molecules and growth factors [1,2]. In solution many proteoglycans are "sticky" and they often form large aggregates if not chromatographed in high molarity denaturing buffers. The high molarity of these denaturing buffers had until recently precluded the analysis of proteoglycans using high-performance liquid chromatography (HPLC) techniques. Although HPLC is commonly used to analyze the detached GAG chains of proteoglycans [3–5], the upper back-pressure limits of most HPLC sizing columns has prevented the analysis of intact proteoglycans under denaturing conditions.

In previous studies, our laboratory has developed and utilized a high pressure anion-exchange chromatographic technique which uses a dissociative buffer [6]. In this study, we were able to take advantage of the anion-exchange technique to partially purify intact proteoglycans from rat chondrosarcoma cells. We then used a BioSil Sec-400 column with an isocratic denaturing mobile phase to approximate molecular mass of intact proteoglycans. The rapid size-exclusion technique is comparable to low pressure conventional techniques because size-

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exclusion chromatography of identical samples using Sepharose CL4B provided very similar molecular mass approximations.

2. Experimental

2.1. Reference compounds and chemicals

The following gel permeation standards were used to calibrate sizing columns: thyroglobulin (M_r 670 000), apoferritin (M_r 443 000), β-amylase (M_r 200 000), bovine serum albumin (M_r 66 000) and ovalbumin (M_r 44 000); the concentration of each standard was 5 mg/ml in 8 *M* urea, 20 m*M* Tris–acetate, pH 6.0. Blue Dextran (M_r 2 000 000) was used to establish V_o; free [^{35}S]sulfate was used at 500 cpm/μl to determine the V_t. All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted.

2.2. Sample preparation

Because 95% of [^{35}S]sulfate is incorporated into the glycosaminoglycan chains of proteoglycans, it is a useful marker to analyze proteoglycan synthesis [7]. Cultures of confluent monolayers of rat chondrosarcoma cells (generously provided by Dr. J. Kimura of Henry Ford Hospital, Detroit, MI, USA) were labeled for 18 h with medium containing [^{35}S]sulfate (Dupont/NEN, Boston, MA, USA) at 100 μCi/ml (3.7 MBq/ml). After labeling, the secreted and cell-associated proteoglycan were harvested as described in [6]. Briefly, the secreted proteoglycans were harvested by removing the culture media and washing the cell layer twice with phosphate buffered saline (PBS). The secreted fraction was dialyzed against 0.1 *M* sodium sulfate and then against a buffer containing 8 *M* urea, 20 m*M* Tris–acetate, pH 5.0. To harvest the cell-associated proteoglycans the remaining cell layer was extracted twice with 4 *M* guanidine hydrochloride in 0.1 *M* sodium acetate buffer, pH 5.8 containing 1 μg/ml pepstatin. The cell-associated fraction was dialyzed against an 8 *M* urea, 20 m*M* Tris–acetate buffer, pH 7.0 containing 0.01% octylglucoside. All harvest solutions and dialysates had the following complement of protease inhibitors (PI): 5 m*M* N-ethylmaleimide, 5 m*M* EDTA, 5 m*M* benzamidine and 2 m*M* phenyl methyl sulfonyl fluoride. Intact proteoglycans were partially purified using anion-exchange high-performance liquid chromatography [6]. Poly-L-glutamic acid (PGA) was added to eluted, pooled proteoglycan samples at a concentration of 0.5 mg/ml before proteoglycan samples were loaded onto the HPLC sizing column.

2.3. Instrumentation and operating conditions

A Dionex BioLC HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Dionex 250×4 mm I.D. ProPac PA1 anion-exchange column was used to resolve heparan sulfate from dermatan/chondroitin sulfate proteoglycans. A Pharmacia 10-ml Superloop (Pharmacia, Uppsala, Sweden) attached to the sample injection port via a Dionex air actuated slider valve (P/N 03598) was used to load 1.5–2.0 ml aliquots from the secreted or the cell-associated proteoglycan fractions. Intact proteoglycans were eluted with a NaCl gradient during a 55-min chromatogram as previously described [6], and proteoglycans labeled with [^{35}S]sulfate were detected using an IN/US on-line radioisotope detector (IN/US Systems, Fairfield, NJ, USA). For HPLC sizing analysis, the Dionex BioLC was equipped with a 300×7.8 mm I.D. Bio-Rad Biosil Sec-400 size-exclusion column (Bio-Rad, Hercules, CA, USA); to protect the integrity of the sizing column, a Biosil Sec-400 guard column (80×7.8 mm I.D.) was installed in front of the sizing column. The stationary phase of the Bio-Rad Biosil Sec-400 size-exclusion column is composed of porous spherical silica beads. The diameter of the beads is 5 μm and the pore size is 400 Å. The beads have a hydrophilic diol bonded-phase to minimize hydrophobic interactions. A 200-μl sample of radiolabeled proteoglycan from the anion-exchange column was loaded onto the Biosil Sec-400 column via a Dionex microinjection valve. The flow-rate during the 1.5-min load was 0.3 ml/min. Proteoglycans were eluted with an isocratic mobile phase of 8 *M* urea, 20 m*M* Tris–acetate, 0.01% octylglucoside, 10 μg/ml PGA, pH 6.0.

Approximately 10% of the eluate from either the anion-exchange or size-exclusion column was ana-

lyzed using the β-RAM IN/US on-line radioisotope detector. The scintillation fluid used was IN-Flow BD (IN/US Systems).

2.4. Sepharose CL4B size-exclusion chromatography

A 90×1.2 cm I.D. Sepharose CL4B column was equilibrated with 8 *M* urea, 20 m*M* Tris–acetate pH 6.0, containing protease inhibitors. The samples were loaded by gravity flow; the flow-rate was 6 ml/h and 1-ml fractions were collected. The V_0 (21.5 ml) and V_t (69 ml) were determined using Blue Dextran 2000 and [^{35}S]sulfate, respectively.

3. Results and discussion

3.1. Preparation of intact proteoglycan samples

Rat chondrosarcoma cells were metabolically labeled with [^{35}S]sulfate. Intact proteoglycans were partially purified using an rapid anion-exchange HPLC procedure previously developed in our laboratory [6]. A NaCl gradient was employed to elute material from the column. Any unincorporated precursor [^{35}S]sulfate elutes just after the gradient begins at ~13.5 ml. Previous studies using cultures metabolically labeled with [^{3}H]serine have shown that cellular proteins elute from the column at NaCl concentrations between ~0.2 and 0.8 *M* [6]. Because proteoglycans are so highly charged, 1.0 to 2.0 *M* NaCl is required to elute this class of macromolecules from the column. This anion-exchange procedure resolves intact proteoglycans into heparan sulfate and dermatan sulfate classes in approximately 55 min.

A representative anion-exchange chromatogram of cell-associated proteoglycans from rat chondrosarcoma cells is depicted in Fig. 1A. The proteoglycan aggrecan is the predominant proteoglycan synthesized by rat chondrosarcoma cells [8,9]; only one distinct peak is normally detected when anion-exchange chromatography is used to analyze the cell-associated fraction from these cells. However, the use of our rapid anion-exchange chromatography procedure in conjunction with an on-line radioisotope detector clearly resolved the cell-associated

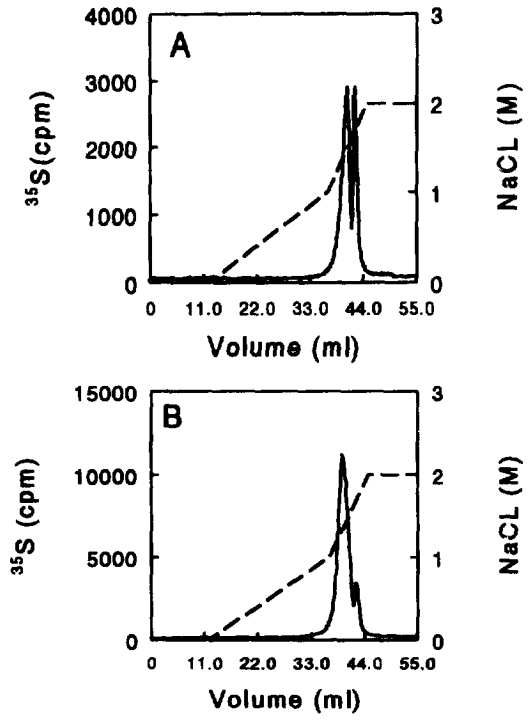

Fig. 1. Rapid anion-exchange HPLC of proteoglycans from secreted and cell-associated fractions from rat chondrosarcoma cells. Panel A shows a representative chromatogram of cell-associated proteoglycans from rat chondrosarcoma cells. Panel B shows a representative chromatogram of secreted proteoglycans from rat chondrosarcoma cells. The dashed line (- - -) represents the NaCl gradient used to elute the proteoglycan fractions. The solid line (–) represents radiolabeled material. Anion-exchange HPLC was performed as described by Blake and McLean [6].

fraction into two peaks. The second peak at 42.4 ml is most likely biglycan and decorin since chondrocytes synthesize minor amounts of these two proteoglycans [10,11]. The assignment of the first peak in the chromatographic profile as aggrecan and the second as biglycan and/or decorin was based on their elution from the column at high ionic strength and the well-documented biosynthetic capabilities of this chondrosarcoma cell line. Unambiguous identification of these peaks could be achieved using antibodies to the respective core proteins; however, such antibodies were not available for these studies. A representative chromatograph of intact proteoglycans from the secreted fraction of rat chondrosarcoma cells is depicted in Fig. 1B. In our hands, a minor peak at 42.4 ml was also resolved in the

secreted fraction. The major peak at 40.3 ml most likely represents aggrecan. Because the putative aggrecan fraction from the cell-associated proteoglycans contains a sufficiently high quantity of intact proteoglycan, this fraction was selected for further sizing studies. Although the secreted fraction has higher quantity of proteoglycan, the cell-associated fraction required a detergent containing buffer and thus, better tested the possible limitations of the column.

3.2. Characterization of Biosil Sec-400 column in high molarity denaturing buffers

High molarity denaturing buffers are required to maintain proteoglycans in an disaggregated state. Because these buffers generate high back-pressure when they are used with HPLC columns, the back-pressure limits of many columns limits their use for the analysis of proteoglycans by HPLC.

The Bio-Rad BioSil Sec-400 size-exclusion column was chosen because it was compatible with high molarity urea buffers used in these analyses and because it could withstand back-pressures of >5 MPa at desired flow-rates. Other available HPLC gel permeation columns were not suitable for these analyses. For example, Dionex produces a gel permeation column, Zorbax, which is not compatible with the high molarity buffers required for the separation of proteoglycans. Pharmacia produces high-performance gel permeation columns which are compatible with 8 *M* urea; however, the pressure limits of these columns are relatively low, ranging from ~1.50 to 1.90 MPa. To use the Pharamacia columns in our system would require lowering flow-rates to 0.25 ml/min or less; the lower flow-rates would increase the time required for size-exclusion chromatography of intact proteoglycans to more than 100 min.

The back-pressure upper limit on the BioSil Sec-400 column is 10.34 MPa. In order to determine maximum flow-rates which could be used with 6 *M* or 8 *M* urea buffers, we investigated the effect of flow-rate on column back-pressures as shown in Fig. 2A. When the 6 *M* or 8 *M* urea was used as an eluate, flow-rates as high as 0.8 ml/min could be attained without reaching the back-pressure limits of the column. Because the 8 *M* urea buffer more

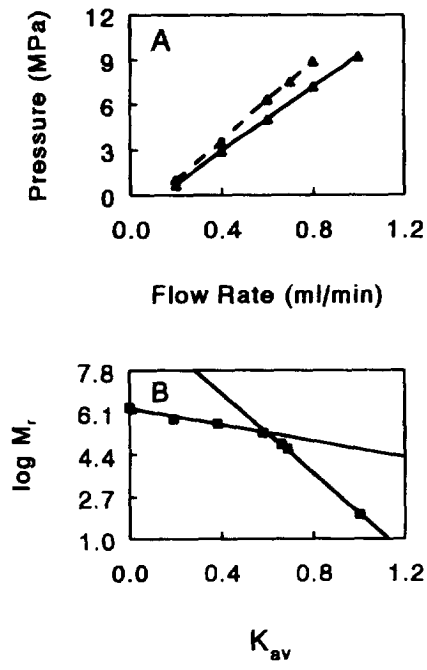

Fig. 2. Characteristics of BioSil Sec-400 column. (Panel A) Flow-rate vs. back-pressure. The back-pressure exerted on the column was monitored at various flow-rates in 6 *M* or 8 *M* urea containing 20 m*M* Tris–acetate buffer, pH 6.0+*P*/s. The upper limit of back-pressure for this column is 10.34 MPa. The dashed line (- - -) represents 8 *M* urea. The solid line represents 6 *M* urea. (Panel B) Calibration curve in 8 *M* urea. The BioSil Sec 400 column was calibrated in 8 *M* urea with the molecular mass standards Blue Dextran (2 000 000), thyroglobulin (669 000), apoferritin (443 000), β-amylase (200 000), bovine serum albumin (66 000), ovalbumin (44 000) and free SO_4 (95.5). The K_{av} for each molecular mass standard ($n=2$) was determined and plotted vs. log M_r.

efficiently maintained the proteoglycan samples in their disaggregated state, the 8 *M* urea buffer at a flow-rate of 0.6 ml/min was used for further sizing studies; the back-pressure exerted on the column was <6.89 MPa when the column was run at a flow-rate of 0.6 ml/min.

Because the high molarity denaturing buffers effect the elution profiles of molecular mass standards, it was necessary to generate a calibration curve for the BioSil Sec-400 in 8 *M* urea. Blue Dextran (M_r 2 000 000) was used to determine V_o and free [^{35}S]sulfate was used to determine V_t. Chromatography standards in an isocratic mobile phase of 8 *M* urea demonstrated that the BioSil

Sec-400 effectively separated proteins from a molecular mass of 670 000 to 44 000. The calibration curve had two linear regions, as shown in Fig. 2B; for higher-molecular-mass samples it was better to use the calibration curve generated with the standards from a molecular mass of 670 000 to 200 000. A second calibration curve was defined using standards from 200 000 to 44 000. To ensure that the high molar denaturing buffers had not changed the properties of the BioSil Sec-400 column, the column was reequilibrated in the low molarity buffer which the manufacturer recommends and a set of molecular mass standards were chromatographed. The chromatographic profile of the standards gave the same profile before and after the use of denaturing buffers (data not shown).

3.3. Rapid size-exclusion chromatography of intact proteoglycans

The nature of intact proteoglycans creates several obstacles to their study using HPLC. In addition to the back-pressure limits that make Pharmacia columns unsuitable for the analysis of intact proteoglycans, the pump systems of some HPLC systems also cannot contend with the pressure generated in the presence of the high molarity buffers. A second obstacle to the study of intact proteoglycans using HPLC sizing columns was that of non-specific binding to the column resins. This non-specific binding to the column may cause the proteoglycan sample to be lost during chromatography. As was discussed previously, the BioSil Sec-400 column can be used at a flow-rate sufficiently high to be expedient as analytical tool without generating back-pressures beyond the limits of the column. To overcome the obstacle of non-specific binding, poly-L-glutamic acid was tested for its ability to reduce non-specific binding of proteoglycan samples. The addition of poly-L-glutamic acid to both the proteoglycan sample and the mobile phase had a striking effect on sample recovery. The inclusion of this polyanionic protein increased sample recovery from ~15% to >80% in replicate samples ($n=4$).

A representative HPLC size-exclusion chromatogram of cell-associated proteoglycans from rat chondrosarcoma is shown in Fig. 3. The major proteoglycan component of the cell-associated fraction is

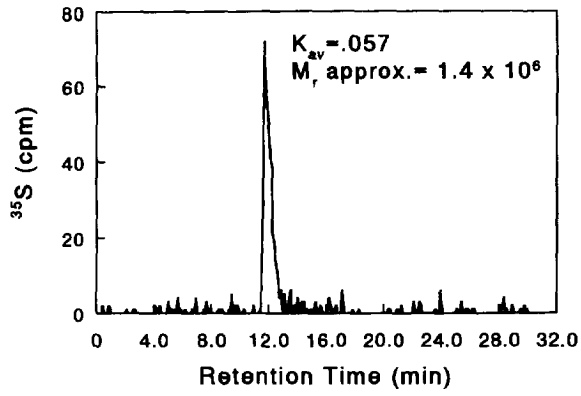

Fig. 3. Rapid size-exclusion chromatography of proteoglycans using BioSil Sec-400. Pooled fractions from the anion-exchange column were resolved on the basis of size using a BioSil Sec-400 column. A 100-μl aliquot of radiolabeled PG sample was loaded and the elution pattern was monitored using a β-RAM on-line radioisotope detector. The figure shows a representative chromatogram of cell-associated proteoglycans from rat chondrosarcoma chromatographed at a flow-rate of 0.6 ml/min in an isocratic system with a buffer containing 8 *M* urea, 20 m*M* Tris–acetate pH 6.0, 0.01% octylglucoside plus PIs and 0.05% sodium azide. Poly-L-glutamic acid (M_r 74 000, 10 μg/ml) was added to the buffer to reduce losses from non-specific binding.

aggrecan. Aggrecan eluted from the column with a retention time of 12 min. The calculated K_{av} was 0.057 which approximates the molecular mass of $1.4 \cdot 10^6$. The remarkable feature of this technique is the speed with which a molecular mass approximation could be obtained. Even when one includes the anion-exchange HPLC step, a molecular mass approximation can be obtained in less than 2 h; with conventional techniques, a molecular mass approximation would take >18 h.

3.4. Comparison of rapid size-exclusion chromatography and conventional size-exclusion chromatography

To compare the BioSil Sec-400 column with conventional low-pressure size-exclusion methods, identical proteoglycan samples were chromatographed on a Sepharose CL4B column. A calibration curve for the Sepharose CL4B column was generated using a selection of molecular mass markers which were similar to the ones chosen for calibration of the BioSil Sec-400 column. As shown in Fig. 4, the

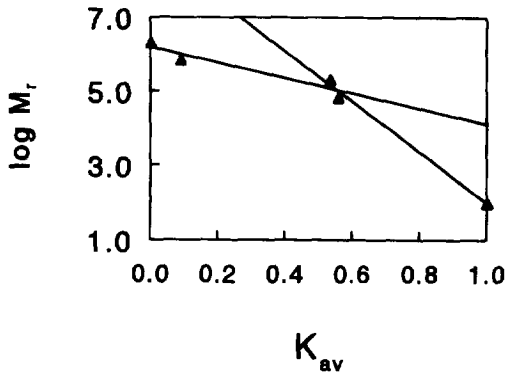

Fig. 4. Calibration curve for Sepharose CL4B column. The Sepharose CL4B column was calibrated in 8 *M* urea with the molecular mass standards Blue Dextran (2 000 000), thyroglobulin (669 000), β-amylase (200 000), bovine serum albumin (66 000) and free SO_4 (95.5). The K_{av} values were determined for molecular mass standards and plotted vs. log M_r.

CL4B calibration curve also had two linear sections: a high-molecular-mass section (670 000–66 000) and a low-molecular-mass section (200 000–100). The higher-molecular-mass section was used to approximate the K_{av} values of the intact proteoglycans synthesized by rat chondrosarcoma cells.

A representative Sepharose CL4B size-exclusion chromatograph of intact cell-associated proteoglycans is shown in Fig. 5. The proteoglycan peak eluted from the column at ~23 ml; the retention time for the peak was approximately 16 h. The K_{av} for the peak was 0.019; although this K_{av} differs from the one generated for the BioSil Sec-400, the molecular mass approximation of $1.3\times\cdot10^6$ was virtually the same for the two columns. To further demonstrate that the two columns have the capacity to generate similar molecular mass approximations, in Fig. 6, the K_{av} values of the molecular mass standards from the BioSil Sec-400 column were plotted with respect to the K_{av} values from the Sepharose CL4B column. This plot was essentially linear, which suggests that the two columns separate molecules by size in a similar manner. It is clear that the BioSil Sec-400 column has the capacity to separate proteins even when high molar denaturing eluates are employed.

The advantages of this rapid size-exclusion technique are obvious; because the samples elute within 25 min in comparison to 18 h for conventional low-pressure size-exclusion chromatography, it is possible to perform very rapid characterizations of metabolically radiolabeled proteoglycans. When using the size-exclusion HPLC technique in conjunction with anion-exchange HPLC, it is possible to separate proteoglycans into dermatan and heparan sulfate classes, and then estimate the molecular size of proteoglycans in the pooled fractions in less than 2 h. In addition to being time-efficient, this HPLC

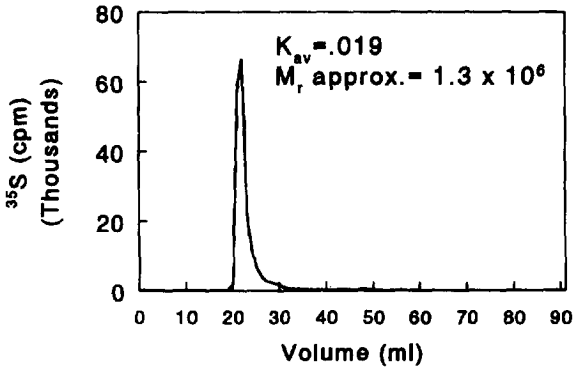

Fig. 5. Size-exclusion chromatography of proteoglycans using Sepharose CL4B. Pooled fractions from the anion-exchange column were resolved on the basis of size using a Sepharose CL4B column and buffers as described in Section 2.4. A 1-ml aliquot of each radiolabeled PG sample (at least 40 000 cpm/ml) was loaded and fractions were collected in 1-ml volumes. A 200-μl aliquot of each fraction was counted in a beta scintillation counter and cpm were plotted with respect to elution volume.

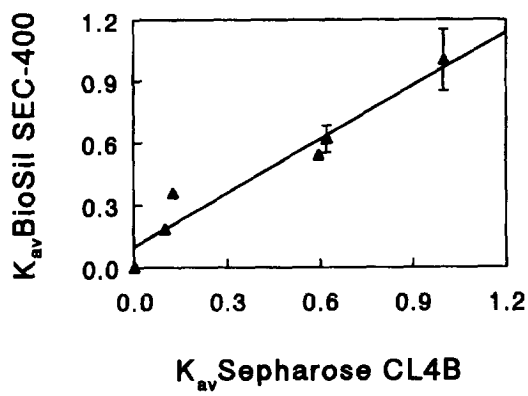

Fig. 6. Comparison between the BioSil Sec-400 column and the Sepharose CL4B column. The molecular mass standards were chromatographed ($n=2$) using the BioSil Sec-400 column. The calculated K_{av} values were then plotted against the K_{av} values determined from Sepharose CL4B chromatography. The linear regression equation is $y=0.87\pm0.11x+0.10\pm0.06$; the correlation coefficient of this line is 0.97.

analysis is also cost effective if many samples must be characterized.

Because proteoglycans are an ubiquitous and multifunctional class of macromolecules which play roles in a number of biological processes, including signal transduction, cell adhesion, and the maintenance of extracellular matrix integrity, structural characterizations of the intact molecules may elucidate our understanding of functional roles. Changes in size or sulfation patterns of proteoglycans may alter the function of these molecules. It has been suggested that metastatic tumor cells inappropriately degrade glycosaminoglycan chains of proteoglycans [12]. Because proteoglycans are involved with the function of a number of growth factors, alterations in proteoglycan structure have been implicated in inappropriate signal transduction via those growth factors [2]. Techniques to structurally characterize proteoglycans should extend the general knowledge base of their roles in biological systems.

Few HPLC techniques for intact proteoglycans have been described in the literature. One technique which was designed to determine molecular size of proteoglycans essentially separated proteoglycan monomers from aggregates using two coupled large-pore silica-based columns [13]. These columns, however, could not tolerate the high ionic strength buffers used in the present study. Because the proteoglycan samples used in our HPLC sizing technique are maintained in high molar chaotropic buffers during sample preparation and chromatographic analysis, the intact proteoglycans should remain as monomers throughout the anion-exchange and sizing analysis. Another HPLC method utilized a silica-based resin bonded with an amide mobile phase to determine molecular size of proteoglycans [14]. An advantage of this previously reported technique was that it did not require dissociative buffers; however, in order to impede the formation of aggregates which commonly form under non-dissociative conditions, the proteoglycan samples required extensive purification (ion-exchange chromatography, gel permeation and sucrose density centrifugation) prior to their application to the HPLC column [15]. Such an isolation process is certainly not trivial. Since the technique developed in our laboratory maintains the proteoglycan samples in a dissociative buffer, the proteoglycans samples need only be separated from the bulk protein using anion-exchange HPLC, a process which takes approximately 55 min, before they can be loaded onto the BioSil Sec-400 HPLC sizing column. Because the combined process of anion-exchange HPLC and the sizing HPLC developed in our laboratory require only 2 h to approximate molecular size of intact proteoglycans, these techniques afford an improvement to other HPLC techniques designed to determine molecular size of intact proteoglycans.

Acknowledgments

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