Analysis of the Proteoglycans Synthesized by Corneal Explants from Embryonic Chicken

II. STRUCTURAL CHARACTERIZATION OF THE KERATAN SULFATE AND DERMATAN SULFATE PROTEOGLYCANS FROM CORNEAL STROMA*

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Radioisotopically labeled proteoglycans were isolated from a 4 M guanidine HCl, 2% Triton X-100 extract of corneal stroma from day 18 chicken embryos by anion-exchange chromatography. Two predominant proteoglycans in the sample were separated by octyl-Sepharose chromatography using a gradient elution of detergent in 4 M guanidine HCl. One proteoglycan had an overall mass of ~125 kDa, a single dermatan sulfate chain (~85–90% chondroitin 4-sulfate, low iduronate content) of ~65 kDa, and a core protein after chondroitinase ABC digestion of ~45 kDa which also contained one to three N-linked oligosaccharides and one O-linked oligosaccharide. The other proteoglycan had an overall size of ~100 kDa, two to three keratan sulfate chains of ~15 kDa each, and a core protein following keratanase digestion of ~51 kDa which included two to three N-linked but no O-linked oligosaccharides. A larger size, a greater overall hydrophobicity (as measured by its interaction with octyl-Sepharose), and an absence of O-linked oligosaccharides argue that this core protein is a distinct gene product from the core protein of the dermatan sulfate proteoglycan.

The preceding paper (1) describes the biosynthesis of the proteoglycans in corneal explant cultures from day 18 chicken embryos. Two predominant proteoglycans were revealed in extracts of corneal stroma from these cultures by using: (i) anion-exchange chromatography to isolate the proteoglycans from other proteins and glycoproteins, and (ii) glycosaminoglycan-degrading enzymes in combination with gel filtration chromatography to identify the proteoglycans. This observation is consistent with information obtained from other animal species (2). These corneal proteoglycans, a keratan sulfate proteoglycan (KS-PG)† and a dermatan sulfate proteoglycan (DS-PG), are of particular interest because of their possible interactions with collagen fibrils (3) which form the theoretical basis of their biological functions. It is thought that these interactions help maintain the precise spacing of the collagen network in the stroma necessary for corneal transparency.

A structural analysis of these proteoglycans is essential to understand the nature of their interactions with collagen as well as their metabolism by corneal stromal fibroblasts. To varying extents, the structural characterization of corneal proteoglycans from monkey (4, 5), bovine (6–8), and rabbit (9, 10) corneal stroma has been performed. The corneal proteoglycans from embryonic chicken have not been structurally analyzed, although the glycosaminoglycans in this tissue have been studied (11, 12). The objective of this investigation was to determine the chemical structure of both KS-PG and DS-PG from the corneal stroma of day 18 chicken embryos.

EXPERIMENTAL PROCEDURES

Materials and methods were as previously described (1), with the addition of the following supplies and procedures.

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Hyaluronic acid (6), and trifluoroacetic acid (both Sequanal-grade) were purchased from Pierce Chemical Co. SDS-PAGE protein standards (14C-methylated) were purchased from Bethesda Research Laboratories.

EXPERIMENTAL PROCEDURES

Materials—Phenyl-Sepharose CL-4B and octyl-Sepharose CL-4B were purchased from Pharmacia LKB Biotechnology Inc. Hydrochloric acid (6 n) and trifluoroacetic acid (both Sequanal-grade) were obtained from Pierce Chemical Co. SDS-PAGE protein standards (14C-methylated) were purchased from Bethesda Research Laboratories.

Hydrophobic Interaction Chromatography—Under optimal conditions, the two predominant proteoglycans of corneal stroma were not resolved adequately on phenyl-Sepharose for this to be a useful separation technique. Instead, octyl-Sepharose was selected for the experiments reported here. To reduce the concentration of CHAPS, samples (0.1 ml) of stromal proteoglycans were diluted 20-fold into 4 M guanidine HCl buffered with 50 mM sodium acetate (pH 6.0) or 8 M urea, 0.15 M NaCl, and 50 mM sodium acetate (pH 6.0) containing 10 µg/ml bovine serum albumin. The samples (±2 µg of proteoglycan protein/µl of column bed) were applied (25 cm/h) to columns of octyl-Sepharose equilibrated in the same guanidine HCl or urea solution (minus the bovine serum albumin carrier). All solutions were treated with activated charcoal to remove impurities that interfere with this chromatography. Some manufactured lots of octyl-Sepharose had high affinity binding sites which irreversibly bound the proteoglycans to the gel; these sites could be blocked using bovine serum albumin (50–100 µg/ml of column bed) prior to sample application. Numerous organic solvents and detergents were tested in order to identify the optimal conditions which elute the bound proteoglycans from the gel. A gradient elution of samples from the octyl-Sepharose columns using 0–1.5% CHAPS in the presence of guanidine HCl or urea solution (30 times the bed volume at 10 cm/h) provided the best resolution between the two major proteoglycans of corneal stroma. The addition of an aliquot of concentrated detergent to the fraction tubes prior to efficient collection prevented adsorption of eluted materials to the tube, particularly materials that eluted below 0.2% CHAPS. Columns eluted with detergents were regenerated by washing with 95% ethanol, followed by 1-butanol prior to

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equilibrium with the initial solvent. 

**Isopycnic CaCl<sub>2</sub> Centrifugation—**Aliquots of DS-PG and KS-PG in 4 M guanidine HCl, 0.5% CHAPS, and 50 mM sodium acetate (pH 6.0) were brought to an initial density of 1.45 g/ml by adding solid CaCl<sub>2</sub>. CaCl<sub>2</sub> density gradients were formed in a Beckman SW 50.1 rotor centrifugation at 37,000 rpm at 10 °C for 48 h. The polyal- 
lomer tubes were punctured at the bottom, and 0.5-ml fractions were 
collected. A small aliquot was removed from each fraction for CaCl<sub>2</sub> 
density determination, and the remainder was assayed for radioactiv-
ity as described previously (1).

**Polyacrylamide Gel Electrophoresis—**Samples of proteoglycans and core protein preparations were electrophoresed on 4–20% linear polyacrylamide gradient gels under reducing conditions in the presence of SDS as described by Fisher et al. (13). Fluorography was performed according to Bonner and Laskey (14).

**Disaccharide Analysis—**Dermatan sulfate chains isolated from DS-
PG by alkaline borohydride treatment and Superose 6 chromatography were digested with chondroitinase ABC as previously described (1). Protein was removed from the resultant digestion products by ethanolic precipitation (15), and chondroitin sulfate disaccharide standards (20 μg each) were then added to the sample. Disaccharides were separated by Partisil 5-PC chromatography according to Ze-
brower et al. (15). Standards were detected by absorbance at 232 nm 
using a Shimadzu SPD-6AV detector. Fractions of 0.5 ml were col-
clected for the determination of radioactivity.

**Molecular Sieve Chromatography—**Oligosaccharides were isolated from alkaline borohydride-treated proteoglycan by Superose 6 chro-
matography as previously described (1). They eluted from DEAE-
Sephacel columns (0.7 x 2.5 cm) in 0.25 M pyridinium acetate (pH 
5.0) without binding, well separated from trace amounts of radioactive 
sulfate in the sample which were partially retarded and from labeled 
glycosaminoglycans which were tightly bound. The recovered olio-
saccharides were then chromatographed (5 ml/h) on a column (0.65 
× 95 cm) of Bio-Gel P-10 in 0.5 M pyridinium acetate (pH 5.0). About 
0.3 mg of chondroitin sulfate C (ICN) and 0.5 mg of glucuronic acid 
lactone were added to each sample to identify the totally excluded 
and included volumes, respectively. Fractions of 0.3–0.4 ml were 
collected and aliquots from each were assayed for radioactivity. 
Total sample recovery was typically >90% of the starting material.

Bio-Gel P-4 chromatography was used to separate chondroitin 
sulfate disaccharides from other oligosaccharides. A column (0.65 
× 95 cm) of Bio-Gel P-4 was eluted (3 ml/h) with 0.5 M pyridinium acetate (pH 5.0). The totally excluded and included volumes of each 
column run were identified as described above for the P-10 columns. 
Chondroitin sulfate disaccharides (~100 μg each) were also added to 
the samples as internal standards. Fractions of 0.3 ml were collected 
and assayed for radioactivity.

**Hexosamine/Hexosaminitol Analysis—**Samples of oligosaccharides 
and glycopeptides were hydrolyzed in 4 M HCl at 100 °C for 10 h to 
release hexosamines (16). Acid was removed under vacuum on a 
Speed Vac concentrator, and the dried samples were then resuspended in 20 μl of 10 mM HCl. Galactosamine and galactosaminitol (4°C-
labeled) were added to the samples as internal standards. Hexosa-
minal standards were prepared by treating hexosamines with 100 
μM NaBH<sub>4</sub> in 50 mM NaOH at 45 °C for 24 h. Hexosamines and 
hexosaminitols were separated on an Aminex A9 column as described by 
Lohmander (16) except that the KHP<sub>4</sub>O<sub>7</sub> concentration was in-
creased to 134 mM. Fractions of 0.3 ml were collected for the deter-
mination of radioactivity. Total sample recovery was 80–85%.

**Aldose/Alditol Analysis—**Samples of glycopeptides labeled with [2-
3H]mannose as precursor were hydrolyzed in 4 M trifluoroacetic acid at 
100 °C for 4 h to released aldoses (16). Acid was removed using a 
Speed Vac concentrator, and the dried samples were then resuspended in 20 μl of water. Glucose and glucitol (both 4°C-labeled) were added to 
the samples as internal standards. Alditols were prepared from 
their aldoses as described above for hexosaminitols. Aldoses and 
alditols were separated on an Aminex 87P column in water at 85 °C. 
Guard columns were used to remove hexosamines and other charged 
sugars from the samples (16). Fractions of 0.25 ml were collected for radioactivity determination. Sample recovery was 85–90% of the start-
ing material. The migration of the following standards relative to 
glucose mobility (set at 1.00) were: fucose, 1.28; mannose, 1.31; 
mannitol, 2.05; and glucitol, 2.85.

**RESULTS**

**Isolation of Corneal Stromal Proteoglycans—**Freshly isol-
ated corneal explants from day 18 chicken embryos were 
labelled in vitro for 6 h with [35S]sulfate and [3H]leucine and 
dissected to liberate stromal tissue according to Midura et al. (1). The radiolabeled macromolecules from the stroma were 
extracted using a 4 M guanidine HCl, 2% Triton X-100 
solution and isolated using Sephadex G-50 chromatography 
in 8 M urea, 0.5% Triton X-100, 0.15 M NaCl, and 50 mM 
sodium acetate (pH 6.0) as previously described (17). These 
labelled macromolecules were applied to a DEAE-Sephacel 
column in this solvent (Fig. 1). Material not bound to the 
DEAE ligand (85% of the 3H radioactivity and 5% of the 35S 
radioactivity) was washed through the column with the start-
ing solvent. Bound macromolecules were released from the 
column during a 0.15–1.0 M NaCl gradient. In this experiment, 
90% of the 35S radioactivity and 10% of the 3H radioactivity 
eluted from DEAE-Sephacel between 0.3 and 0.6 M NaCl 
(indicated by the bar in Fig. 1), where the vast majority of 
corneal proteoglycans have been reported to elute (1).

The proteoglycan pool was applied to a column of octyl-
Sephacel in 4 M guanidine HCl, and a majority of the 
radioactivity bound to this hydrophobic matrix (Fig. 2). The 
unbound material contained only 8% of the 35S activity and
Identification of Proteoglycans—An aliquot of Pool 1 from octyl-Sepharose eluted on Superose 6 in dissociative conditions as a single peak with a $K_d$ of 0.25 ($\sim 125$ kDa) (Fig. 3A). Chondroitinase ABC digested 98% of the $^{35}S$ activity of Pool 1 into disaccharides while liberating a single core protein peak ($K_d = 0.57$; $\sim 60$ kDa) containing virtually all of the $^3H$ activity and the remaining 2% of the $^{35}S$ activity (Fig. 3B). The bound material as two peaks during a gradient of the detergent CHAPS in the guanidine HCl solvent. The first peak contained 45 and 49% of the $^{35}S$ and $^3H$ activity, respectively, whereas the second peak had 46 and 49%.

Pool 2 chromatographed as a single peak of $K_d = 0.35$ ($\sim 100$ kDa) (Fig. 4A). Keratanase digested 95% of the total $^{35}S$ activity from Pool 2 into disaccharides and larger oligosaccharides while liberating a single core protein peak ($K_d = 0.57$; $\sim 60$ kDa) containing 85% of the $^3H$ activity and 2% of the $^{35}S$ activity (perhaps as a residual disaccharide not removed by the enzyme) (Fig. 4B). The remaining 5% of the $^{35}S$ and $^3H$ activity eluted at $K_d = 0.25$. This represents cross-contaminating DS-PG from Pool 1 for the following reasons. (i) Digestion of Pool 2 material by chondroitinase ABC released $\sim 5%$ of the $^{35}S$ activity as disaccharides (data not shown); (ii) it can be removed by a second application of Pool 2 to octyl-Sepharose (data not shown); (iii) it has the same hydrodynamic size as Pool 1 proteoglycan; and (iv) it has the same glycosaminoglycan size (see Fig. 7) and disaccharide composition (data discussed below) as for DS-PG from Pool 1.

Cesium chloride density gradient centrifugation of these DS-PG and KS-PG preparations in 4 M guanidine HCl yielded broad, unimodal profiles centering about 1.42 g/ml (Fig. 3A, inset) and 1.4 g/ml (Fig. 4A, inset), respectively. These buoyant densities would indicate protein contents of $\sim 50\%$ (19).

Nature of Proteoglycan Interaction with Octyl-Sepharose—When chondroitinase ABC or keratanase digests were applied to octyl-Sepharose, the core protein preparations from both DS-PG and KS-PG bound and eluted with identical characteristics as for their respective proteoglycans (Fig. 5, A and B). Neither the disaccharide and oligosaccharide digestion products (Fig. 5) nor the glycosaminoglycan chains of either proteoglycan released by papain or alkaline borohydride treatment (data not shown) bound to the matrix under such conditions. Thus, the core proteins determine the interactive properties of these proteoglycans with the octyl ligand.

Molecular Mass Determination by SDS-PAGE—Both proteoglycans and their core protein preparations were subjected to electrophoresis on a 4–20% polyacrylamide gradient slab gel in SDS and detected by subsequent fluorography (Fig. 6). The intact proteoglycans appeared as extremely broad bands with each having a median mass of $\sim 200$ kDa when compared to globular protein standards. These values are overestimates.

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**Fig. 3.** Superose 6 elution profiles of first proteoglycan pool from octyl-Sepharose indicated in Fig. 2. A, untreated sample (inset depicts the distribution of this sample in a CsCl density gradient); B, sample after chondroitinase ABC digestion; C, glycosaminoglycans isolated from an alkaline borohydride digest of the sample and then digested with chondroitinase AC II (arrow points to oligosaccharides).

**Fig. 4.** Superose 6 elution profiles of second proteoglycan pool from octyl-Sepharose indicated in Fig. 2. A, untreated sample (inset depicts the distribution of this sample in a CsCl density gradient); B, sample after keratanase digestion.

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of the actual molecular masses for these proteoglycans since other techniques such as sedimentation equilibrium centrifugation (6, 7) and molecular sieve chromatography (Figs. 3A and 4A) indicate masses of about one half those observed in Fig. 6. In a few experiments, these proteoglycans migrated as though their median molecular masses were ~140 kDa (not not shown). This variable migration indicates that they exhibit anomalous behavior on SDS-PAGE.

The core protein preparation of DS-PG migrated as one major band at 45 kDa and two minor ones at 43 and 40 kDa. Absorbance scanning of the fluorogram indicated that ~85% of the sample radioactivity resided in the major band. The core protein preparation of KS-PG migrated as a single band at 51 kDa, distinctly larger than for the DS-PG core protein.

Glycosaminoglycan Analyses—Corneal explants from day 18 chicken embryos were incubated with [35S]sulfate and [3H]glucosamine to label the glycosaminoglycans and oligosaccharides of the proteoglycans. Labeled DS-PG and KS-PG were isolated from the stromal tissue as described above. Their glycoconjugates were liberated by alkaline borohydride treatment and then eluted on Superose 6 to separate the glycosaminoglycans from smaller oligosaccharides. This treatment releases O-glycosidically linked glycoconjugates via a β-elimination reaction and extensively hydrolyzes peptide bonds in core proteins, thereby releasing N-linked glycopeptides (20–22). Consistent with this fact, papain treatment of each proteoglycan in this study gave virtually identical results as for the respective alkaline borohydride treatment (data not shown) similar to what had been established for corneal proteoglycans from monkey (4).

The Superose 6 profile for the sample from DS-PG revealed a major dermatan sulfate peak \( (K_d = 0.31; \sim 65 \text{ kDa}) \) with 95% of the \( ^{35} \text{S} \) activity and 87% of the \( ^{3} \text{H} \) activity (Fig. 7A). Digestion with chondroitinase ABC yielded disaccharides that were primarily 4-sulfated (87%), with modest amounts of nonsulfated and 6-sulfated species as well as a small amount of disulfated disaccharide (Table I). An oligosaccharide peak eluted at \( K_d = 0.94 \) and contained approximately 8% of the \( ^{3} \text{H} \) activity, but virtually no \( ^{35} \text{S} \) activity (Fig. 7A). These oligosaccharides were pooled for further analysis as described below.

The Superose 6 profile for the sample from KS-PG revealed a major keratan sulfate peak \( (K_d = 0.67; \sim 15 \text{ kDa}) \) with about 92% of the \( ^{35} \text{S} \) activity and 85% of the \( ^{3} \text{H} \) activity (Fig. 7B). The small peak at \( K_d = 0.31 \) is dermatan sulfate derived from the 5% cross-contamination of this preparation with DS-PG described above. A small amount of \( ^{35} \text{S} \) activity eluted near the totally included volume of the column and is probably free sulfate released from keratan sulfate by the alkaline treatment (24). The resulting 10% of the \( ^{3} \text{H} \) activity in the profile resolved as a shoulder trailing from the keratan sulfate peak \( (K_d > 0.85) \). These oligosaccharides were pooled for further analyses.

Oligosaccharide Analyses—The recovered oligosaccharide peaks were applied to anion-exchange columns to remove trace amounts of glycosaminoglycans and any free sulfate prior to application on Bio-Gel P-10. The oligosaccharides from DS-PG resolved into four distinct peaks (Fig. 8A). Two of these were relatively large in size \( (K_d = 0.10 \text{ and } 0.38) \) and

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3 Chondroitin sulfate standards were used to calibrate the Superose 6 column and confirm the equation to determine glycosaminoglycan mass: In (mass) = \((3.109 - K_d)/0.253\) reported by Wasteson (23).
Corneal Proteoglycan Structure

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Superose 6

Bio-Gel P-10

FIG. 7. Superose 6 elution profiles of alkaline borohydride-treated DS-PG (A) and KS-PG (B). Explant cultures were labeled with [35S]sulfate and [3H]glucosamine. Bars indicate fractions pooled for oligosaccharide analysis.

Table I

Disaccharide analysis of the dermatan sulfate glycosaminoglycan

A portion of the dermatan sulfate chains from Fig. 7A were digested with chondroitinase ABC and then subjected to Partisil 5-PAC chromatography.

| Sample          | GlcN | GlcN-ol | GalN | GalN-ol | Unbound material |
|-----------------|------|---------|------|---------|-----------------|
|                 | %    | %       | %    | %       | %               |
| DS-PG GAG       | ND   | ND      | 95   | ND      | 5               |
| Oligo Pool 1    | 89   | 3       | ND   | ND      | 8               |
| Oligo Pool 2    | 91   | ND      | ND   | ND      | 9               |
| Oligo Pool 3    | 35   | ND      | ND   | 39      | 26              |
| KS-PG GAG       | 92   | ND      | ND   | ND      | 8               |
| Oligo Pool 1    | 92   | ND      | ND   | ND      | 8               |
| Oligo Pool 2    | 93   | ND      | ND   | ND      | 7               |

*Samples contained the following amounts of recovered [3H] radioactivity: DS-PG GAG (2300 cpm); oligo Pool 1 (430 cpm), oligo Pool 2 (1400 cpm), and oligo Pool 3 (380 cpm); KS-PG GAG (2100 cpm), oligo Pool 1 (1100 cpm), and oligo Pool 2 (800 cpm).

The oligosaccharides from KS-PG eluted as a broad distribution of relatively large molecules on Bio-Gel P-10 (Fig. 8B).
An apparent peak at $K_d = 0.08$ contained 56% of the $^3$H activity, whereas a shoulder appearing off the descending side of this peak ($K_d = 0.20-0.45$) contained about 40%. The remaining 4% of the radioactivity trailed from this shoulder without a detectable peak in the size range of the smaller oligosaccharides from DS-PG. The apparent peak and shoulder material were recovered for hexosamine analysis (Table II). All of the labeled hexosamine in these two pools was in the form of glucosamine, and no galactosaminitol was detected. These data indicate that KS-PG lacks oligosaccharides that are O-linked to the core protein via galactosamine.

The nature of the linkage structure of the larger oligosaccharides from both proteoglycans was further studied by labeling experiments using [2-3H]mannose as a precursor which would be incorporated into all N-glycosidically linked structures. Corneal explants from day 18 chicken embryos were labeled with [2-3H]mannose, and DS-PG and KS-PG from the stromal tissue were prepared as described above. Glycosaminoglycans and oligosaccharides were released from each proteoglycan preparation by alkaline borohydride treatment, followed by elution on a Bio-Gel P-10 column without prior separation on Superose 6, (Fig. 8, C and D). The DS-PG Pool 1 material described below was further processed by chondroitinase ABC digestion and Bio-Gel P-4 chromatography to remove dermatan sulfate and yield just oligosaccharides. Subsequent neutral sugar analysis of all of the peaks revealed that the label was predominantly (>90%) in mannose, with none detected in mannitol (Table III). The lack of mannitol after borohydride reduction shows that the reducing terminal sugar of the oligosaccharides could not be mannose. This indicated that these glycoconjugates were not O-linked via mannose, as has been described for some oligosaccharides and in glycosaminoglycans isolated from brain tissue (25).

The sample from DS-PG revealed two peaks of relatively large size (Fig. 8C). The peak in the void volume contained 20% of the $^3$H activity, one third of which (~7% of the total) was released as disaccharides by chondroitinase ABC diges-

**TABLE III**

Adlase and aldito analysis of oligosaccharides

| Oligosaccharides indicated in Fig. 8 (A and D) were acid-hydrolyzed and then analyzed using Aminex 57E chromatography. Charged sugars were removed from the samples using anionic and cationic guard columns (<5% of the label bound to these guards). | % | % | % |
|---|---|---|---|
| DS-PG | Mannose | Mannitol | Unidentifieda |
| Pool 1 | 100 | NDa | ND |
| Pool 2 | 91 | ND | 9 |
| KS-PG | Mannose | Mannitol | Unidentifieda |
| Pool 1 | 98 | ND | ND |
| Pool 2 | 100 | ND | ND |

a Samples contained the following amounts of recovered $^3$H radioactivity: DS-PG Pool 1 (300 cpm) and Pool 2 (2400 cpm); KS-PG Pool 1 (600 cpm) and Pool 2 (1100 cpm).

b Material had a relative mobility to glucose of 1.5, thus eluting differently from the standards described under "Experimental Procedures."

c Pool 1 from DS-PG was treated with chondroitinase ABC and then chromatographed on Bio-Gel P-4 to remove any radioactivity associated with the liberated disaccharides. The remaining oligosaccharides, all of which eluted in the column's void volume, were recovered for neutral sugar analysis.

Radioactivity in the disaccharides probably reflects minor labeling contaminants in the [2-H]mannose stock (21). Assuming that this contaminant would label galactose pools, it can be calculated from the chemical structures of the glycosaminoglycan and oligosaccharides on DS-PG that the labeling efficiency of galactose relative to mannose is less than 0.5%.

**DISCUSSION**

Keratan sulfate and dermatan sulfate proteoglycans are synthesized and deposited in the stromal tissue of corneal...
explants from embryonic chicken. Both of these proteoglycans are relatively small in size and contain about 50% protein by mass. DS-PG has an intact size of \(-125\) kDa and a single dermatan sulfate chain of \(-65\) kDa that is composed mainly of chondroitin 4-sulfate.\(^6\) This DS-PG also has one to three \(N\)-linked oligosaccharides and one \(O\)-linked oligosaccharide which can be either a hexasaccharide or a tetrasaccharide. Its core protein preparation yielded a major band at 45 kDa with minor bands at 43 and 40 kDa. Glossi et al. (30) showed that a skin fibroblast DS-PG yielded multiple protein bands in this size range after chondroitinase ABC digestion and presented evidence that the different bands represented the same protein with a different number of \(N\)-linked oligosaccharides. It is likely that the same explanation applies for the multiple bands in the DS-PG core preparation from cornea. Accordingly, the completely deglycosylated core protein would be somewhat less than 40 kDa. Based on peptide mapping analysis of a similar DS-PG from bovine cornea (31) and Northern analysis of bovine corneal RNA using a PG-II cDNA probe (32), this proteoglycan is thought to belong to a class of small DS-PG referred to as PG-II (2). However, it is possible that this preparation may contain significant amounts of a closely related DS-PG designated as PG-I which yields a similar sized core protein after chondroitinase digestion (33, 34).

The other predominant proteoglycan in chicken cornea has an intact size of \(-100\) kDa and two to three keratan sulfate chains of \(-15\) kDa that are \(N\)-linked to the core protein. Additionally, it contains two to three \(N\)-linked oligosaccharides whose structures may be similar to those identified in KS-PG from monkey cornea (28). Unlike DS-PG, this proteoglycan has no \(O\)-linked oligosaccharides. Its core protein preparation yields a single band of \(-51\) kDa. The completely deglycosylated core would be smaller, perhaps about 42–45 kDa assuming that a few oligosaccharides still remain attached to the core preparation after keratanase digestion. Therefore, the structures of both DS-PG and KS-PG from the corneal stroma of embryonic chicken appear to be very similar to those of the proteoglycans isolated from bovine (6–8, 35), rabbit (9, 10), monkey (4), and human (36, 37) corneal tissue.

Unlike ion-exchange chromatography or isopycnic centrifugation, hydrophobic interaction chromatography using octyl-Sepharose fractionates proteoglycans according to the chemistry of the constituent polypeptide and not that of the attached glycosaminoglycans. This method has the advantage of separating corneal proteoglycans without enzymatically removing their glycosaminoglycans, which may also expose their core proteins to potential proteolysis from contaminants in the enzyme preparations. Here, this procedure showed that the core protein of KS-PG is more hydrophobic than, and thus chemically different from, that of DS-PG. A larger size and different chemistry argue that it is a separate gene product from the DS-PG core protein. This hypothesis is supported by an absence of immunological identity between the core proteins of these two proteoglycans from monkey and human cornea (5, 38). Whereas the hydrophobicity of core proteins may impart specific conformations vital to the function of proteoglycans in tissues, it remains to be determined whether the greater hydrophobicity of the core protein from KS-PG in comparison to that of DS-PG has a functional significance in the organization of the corneal stroma.

In addition to separating the major proteoglycan species from cornea, other applications of octyl-Sepharose chromatography have recently been demonstrated. Hydrophobic peptide domains of a transferrin-binding heparan sulfate proteoglycan from skin fibroblasts (38) as well as heparan sulfate proteoglycan from colon carcinoma cells (39) have been studied using this technique. A heparan sulfate proteoglycan from aortic endothelial cells which exhibits anticoagulant activity has also been isolated by this procedure (40). PG-I and PG-II from cultured bone cells have been partially fractionated from each other by octyl-Sepharose chromatography (41).

**References**

1. Midura, R. J., Toledo, O. M. S., Yanagishita, M., and Hascall, V. C. (1989) J. Biol. Chem. 264, 1414–1422
2. Hassell, J. R., Kimura, J. H., and Hascall, V. C. (1986) Annu. Rev. Biochem. 55, 539–567
3. Scott, J. E., and Haigh, M. (1980) Biosci. Rep. 5, 765–774
4. Hassell, J. R., Newsome, D. A., and Hascall, V. C. (1979) J. Biol. Chem. 254, 12346–12354
5. Nakazawa, K., Hassell, J. R., Hascall, V. C., and Newsome, D. A. (1983) Arch. Biochem. Biophys. 225, 105–116
6. Axelson, I., and Heinegård, D. (1978) Biochem. J. 169, 517–530
7. Axelson, I., and Heinegård, D. (1980) Exp. Eye Res. 31, 57–66
8. Conrad, G. W., Ager-Johnson, P., and Woo, M.-L. (1982) J. Biol. Chem. 257, 464–471
9. Gregory, J. D., Cöster, L., and Damle, S. P. (1982) J. Biol. Chem. 257, 6965–6970
10. Cöster, L., Cintron, C., Damle, S. P., and Gregory, J. D. (1983) Exp. Eye Res. 36, 517–530
11. Conrad, G. W., and Dorfman, A. (1974) Exp. Eye Res. 18, 421–433
12. Hart, G. W. (1976) J. Biol. Chem. 251, 6513–6521
13. Fisher, L. W., Termine, J. D., Dejter, S. W., Jr., Whitson, S. W., Yanagishita, M., Kimura, J. H., Hascall, V. C., Kleinman, H. K., Hassell, J. R., and Nilsson, B. (1983) J. Biol. Chem. 258, 6538–6549
14. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88
15. Zelazny, M. E., Kieras, F. J., and Brown, W. T. (1986) Anal. Biochem. 157, 93–99
16. Lohmander, L. S. (1986) Anal. Biochem. 154, 75–84
17. Yanagishita, M., Midura, R. J., and Hascall, V. C. (1987) Methods Enzymol. 138, 279–289
18. Hascall, V. C., Riozo, R. L., Hayward, J., Jr., and Reynolds, C. C. (1979) J. Biol. Chem. 247, 4521–4528
19. Hascall, V. C., and Riozo, R. L. (1972) J. Biol. Chem. 247, 4529–4538
20. Yanagishita, M., and Hascall, V. C. (1979) J. Biol. Chem. 254, 12355–12364
21. Lohmander, L. S., De Luca, S., Nilsson, B., Hascall, V. C., Caputo, C. B., Kimura, J. H., and Heinegård, D. (1980) J. Biol. Chem. 255, 6984–6991
22. Nilsson, B., De Luca, S., Lohmander, L. S., and Hascall, V. C. (1982) J. Biol. Chem. 257, 10920–10927
23. Wasteson, Å. (1971) J. Chromatogr. 59, 87–97

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\(^6\) The number of glycosaminoglycans and oligosaccharides per core protein were calculated using the following formulas. (i) Number of glycosaminoglycan chains/proteoglycan = (mass of proteoglycan molecule) / (mass of core protein preparation including oligosaccharides)/([mass/glycosaminoglycan chain]). Using \([^3]H\) glucosamine as precursor; (ii) number of \(O\)-linked oligosaccharides/glycosaminoglycan = ([radioactivity in galactosaminitol of \(O\)-linked oligosaccharides]/[radioactivity in hexosamine of glycosaminoglycan with known mass]) × (number of hexosamines in this glycosaminoglycan). (iii) Number of \(N\)-linked oligosaccharides/glycosaminoglycan = ([radioactivity in hexosamine of \(N\)-linked oligosaccharides of known structure]/[radioactivity in hexosamine of glycosaminoglycan with known mass]) × (number of hexosamines in this glycosaminoglycan). *Assumption must be made whether high mannose or complex-type.
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24. Sampson, P., and Meyer, K. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 2329–2331
25. Krusius, T., Finne, J., Margolis, R. K., and Margolis, R. U. (1986) J. Biol. Chem. 261, 8237–8242
26. Bray, B., Lieberman, R., and Meyer, K. (1967) J. Biol. Chem. 242, 3373–3380
27. Nilsson, B., Nakazawa, K., Hassell, J. R., Newsome, D. A., and Hascall, V. C. (1983) J. Biol. Chem. 258, 6056–6063
28. Stuhlsatz, H. W., Kisters, R., Wollmer, A., and Greiling, H. (1971) Hoppe-Seyler’s Z. Physiol. Chem. 352, 289–303
29. Hart, G. W., and Lennarz, W. J. (1978) J. Biol. Chem. 253, 5795–5801
30. Gläsle, J., Beck, M., and Kresse, H. (1984) J. Biol. Chem. 259, 14144–14150
31. Heinegård, D., Björne-Persson, A., Cöster, L., Franzén, A., Gardell, S., Malmström, A., Paulsson, M., Sandfalk, R., and Vogel, K. (1985) Biochem. J. 230, 181–194
32. Day, A. A., Ramis, C. I., Fisher, L. W., Gehron-Robey, P., Termine, J. D., and Young, M. F. (1986) Nucleic Acids Res. 14, 9861–9876
33. Rosenberg, L. C., Choi, H. U., Tang, L.-H., Johnson, T. L., Pal, S., Wehber, C., Reiner, A., and Poole, A. R. (1985) J. Biol. Chem. 260, 6304–6313
34. Vogel, K. G., and Fisher, L. W. (1986) J. Biol. Chem. 261, 11334–11340
35. Hassell, J. R., Hascall, V. G., Ledbetter, S., Caterson, B., Thonar, E., Nakazawa, K., and Krachmer, J. (1984) in Proceedings of the 8th Symposium on Ocular and Visual Development (Hilfer, S., and Sheffield, J., eds) pp. 101–114, Springer-Verlag, New York
36. Nakazawa, K., Hassell, J. R., Hascall, V. C., Lohmander, L. S., Newsome, D. A., and Krachmer, J. (1984) J. Biol. Chem. 259, 13751–13757
37. Klintworth, G. K., and Smith, C. F. (1983) Lab. Invest. 48, 603–612
38. Fransson, L.-Å., Cöster, L., Carlstedt, I., and Malmström, A. (1985) Biochem. J. 231, 683–687
39. Iozzo, R. V., Ketterer, C. L., and Slaymaker, D. J. (1986) FEBS Lett. 206, 304–308
40. Marcum, J. A., Atha, D. H., Fritz, L. M. S., Nawroth, P., Stern, D., and Rosenberg, R. D. (1986) J. Biol. Chem. 261, 7507–7517
41. Beresford, J. N., Fedarko, N. S., Fisher, L. W., Midura, R. J., Yanagishita, M., Termine, J. D., and Gehron-Robey, P. (1987) J. Biol. Chem. 262, 17164–17172