Purification of Keratan Sulfate Proteoglycan from Monkey Cornea

ISOLATION OF THE KERATAN SULFATE LINKAGE REGION AND THE MANNOSE-CONTAINING OLIGOSACCHARIDES*

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An explant culture of 15 cynomolgus monkey corneas was incubated with [35S]sulfate and [2-3H]mannose as labeling precursors. A 4 M guanidine HCl extract of the corneal stromas was prepared and combined with a 4 M guanidine HCl extract of stromas from 300 unlabeled corneas. The keratan sulfate proteoglycans in the combined extracts were purified by a combination of DEAE-cellulose chromatography, chondroitinase ABC digestion to remove chondroitin-dermatan sulfate proteoglycans, and elution from immobilized concanavalin A. The purified keratan sulfate proteoglycan was digested with papain and the digest was eluted on DEAE-Sephacel. The unbound fraction contained 59% of the 3H activity and consisted of intact oligosaccharide-peptides. The bound fraction, consisting of keratan sulfate chains linked to peptides, eluted during a linear 0-0.75 M NaCl gradient as a peak centered at ~0.6 M NaCl and contained 41% of the 3H and all of the 35S activity in the original proteoglycan. The chains were digested with endo-β-galactosidase, and the digest was eluted on DEAE-Sephacel with a linear 0-0.75 M NaCl gradient. Most of the sulfated digestion fragments from the chains eluted at several distinct peaks during the gradient. All the 3H activity eluted in the unbound volume along with a small proportion of the 35S activity. This unbound fraction was eluted on Bio-Gel P-10 to give a 3H peak (Kav = 0.46) well resolved from the remaining 35S activity which eluted near the total volume. This 3H peak contained the oligosaccharide-peptides derived from the linkage region between the keratan sulfate chains and the core protein. Structural analyses of the linkage region oligosaccharides and the intact oligosaccharides (Nilson, B., Nakazawa, K., Hassell, J. R., Newsome, D. A., and Hascall, V. C. (1983) J. Biol. Chem. 258, 6056-6063) in combination with the 3H-labeling data suggest that the intact keratan sulfate proteoglycans contain an average of about one intact oligosaccharide per keratan sulfate linkage site.

Glycosaminoglycan side chains of proteoglycans are joined to the protein core through unique sequences of carbohydrates which constitute linkage regions. The structure of the linkage region in chondroitin (dermatan) sulfate proteoglycans is

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Experimental Procedures

MATERIALS—Chondroitinase ABC (Proteus vulgaris) and endo-β-
galactosidase (keratanase, Pseudomonas sp.) were purchased from
Miles Laboratories; papain (twice crystallized) was from Sigma
Chemicals; [2-3H]mannose (17.3 Ci/mmol) and [35S]sulfate (100 Ci/mmol) were
from New England Nuclear; DEAE-cellulose (DE52) was from
Whatman; DEAE-Sepharose and ConA-Sepharose were from Phar-
maida; Bio-Gel P-10 was from Bio-Rad. Aqueous counting scin-
tillants (ACS and Aquasol) were purchased from Amersham Corp.
Radioisotope Analyses—Small samples in 4 ml guanidine HCl (20
μl or less) were counted directly in 10 ml of ACS or Aquasol. Larger
aliquots (50-500 μl) were diluted with an equal volume of 70% ethanol
before the addition of scintillants. Samples (5-500 μl) in other solvents
were counted directly in the scintillants. A Beckman LS-385 scinti-
lation counter with a dual label program was used for isotope analyses.

Corneas—Three hundred corneas were obtained from freshly enu-
cleated eyes of cynomolgus monkeys which were being killed for other
purposes. The corneas were immediately frozen and stored at
-70°C until extraction.

Labeling of Corneas with [2-3H]Mannose and [35S]Sulfate—Fif-
teen corneas were extracted with intact scleral rims and were incubated in
CooN's modified Ham's F-12 medium (1 ml/cornea) containing 5%
fetal calf serum, 100 units/ml of penicillin, and 2 μg/mL insulin. The
expiants were incubated with [2-3H]mannose (300 μCi/ml) and [35S]
sulfate (600 μCi/ml) for 18 h. The corneas were then removed from the
medium, centrifuged, rinsed briefly in 2-3 volumes of cold phosphate-buffered saline. They were then trimmed to
remove the scleral rims, rapidly frozen, and stored at -70°C until extraction.

Extraction and Isolation of Keratan Sulfate Proteoglycan—Cor-
neas were individually thawed on ice, rapidly scraped free of epithe-
lium and endothelium, and placed in 4 ml guanidine HCl containing
0.01 M sodium EDTA, 0.01 M sodium acetate, 0.1 M 6-aminoheptanoic
acid, and 0.005 M benzamidase HCl, pH 5.8 (25). The 300 unlabelled
corneas were extracted with 200 ml of the 4 ml guanidine HCl solution for
20 h at 4°C. The extract was decanted, and the residual tissue was
re-extracted in 100 ml of 4 ml guanidine HCl containing the same
protease inhibitors for an additional 40 h at 4°C. The two extracts were
combined and concentrated to about 130 ml by ultrafiltration
with a PM-10 membrane (Amicon) followed by dialysis against 8 m
urea in 0.05 M Tris-HCl, pH 6.8. The dialyzed extracts, containing the 300 unlabelled corneas and the 15 radioabeled
corneas were combined and used for isolation of the keratan sulfate
proteoglycans.

The combined extract was applied to a DEAE-cellulose column
(2.5 x 25 cm) equilibrated with 8 m urea, 0.05 M Tris-HCl, pH 6.8
(26). The column was washed with 285 ml of 8 m urea, 0.05 M Tris-
HCl, pH 6.8. The bound material was then eluted from the column with a
linear gradient of 0-0.75 M NaCl (total 800 ml) in the same solvent, and then
with 80 ml of 3.0 M NaCl in the urea solvent. Fractions of 8.5 ml were
collected. The main bound peak containing both 3H and 35S activity
was pooled as described in Fig. 2 below, concentrated by ultrafiltration
with an Amicon PM-10 membrane, and dialyzed against 1 M NaCl,
0.05 M Tris-HCl, pH 7.0.

The dialyzed fraction was applied to a concanavalin A-Sepharose column
(2.5 x 10 cm) equilibrated with 1 M NaCl, 0.05 M Tris-HCl, pH 7.0. The column was washed with 390 ml of 1 M NaCl, 0.05 M Tris-
HCl, pH 7.0, and the bound material was eluted with 390 ml of 1 m
sodium acetate, 1 M Tris-HCl (pH 7.0). Fractions of 16 ml were collected. The ConA-bound fraction, containing 3H and 35S activity, was pooled as described in Fig. 3 below, dialyzed against distilled water, and lyophilized (yield, ~80 mg). This ConA-bound fraction contained purified keratan sulfate proteoglycans.

Isolation of the Keratan Sulfate Linkage Region Oligosaccharide
and of Mannose-containing Oligosaccharide—All of the purified
keratan sulfate proteoglycan fraction was dissolved in 3 ml of 1 m
sodium acetate, pH 6.5, containing 5 mM sodium EDTA and 5 mM
mercaptoethanol, and digested with papain at 55°C for
7 h. The reaction was terminated with the addition of 6 mg of iodo-
acetamide. The digest was diluted 10-fold with water and applied to
a column of DEAE-Sepharose (1.6 x 6.5 cm) equilibrated with 0.1 m
sodium acetate, pH 7.0. The column was washed with 100 ml of 0.1 m
sodium acetate, pH 7.0. The bound material was eluted with a
linear gradient of 0-0.2 M NaCl in 0.1 M sodium acetate, pH 7.0, and then with 40 ml of 3.0 M NaCl in 0.1 m sodium acetate, pH 7.0. Fractions of 4 ml were collected. The unbound fractions, containing 3H activity, and the bound fractions, containing both 3H and 35S activity were pooled, respectively, as described in Fig. 4 below. The unbound fraction, which contained intact glycopeptides, was directly lyophilized and subsequently treated as described below.

The bound fraction, containing purified keratan sulfate chains, was
lyophilized after dialysis against distilled water.

The keratan sulfate chain fraction (72 mmol as galactose) was digested with 0.6 m of 0.05 m Tris-HCl, pH 7.5, and then with 250 ml of 8 m urea, 0.05 M Tris-HCl, pH 7.5 (27). The reaction was terminated with the addition of 6 mm of iodo-
acetamide. The digest was diluted 10-fold with water and applied to
a column of DEAE-Sepharose (1.6 x 6.5 cm) equilibrated with 0.1 m
sodium acetate, pH 7.0. The column was washed with 100 ml of 0.05 m
sodium acetate, pH 7.0. The bound material was eluted with a
linear gradient of 0-0.2 M NaCl in 0.1 M sodium acetate, pH 7.0, and then with 40 ml of 3.0 M NaCl in 0.1 m sodium acetate, pH 7.0. Fractions of 4 ml were collected. The unbound fractions, containing both 3H and 35S activity were pooled, respectively, as described in Fig. 4 below. The unbound fraction, which contained intact glycopeptides, was directly lyophilized and subsequently treated as described above.

The bound fraction, containing purified keratan sulfate chains, was
lyophilized after dialysis against distilled water.

RESULTS

Isolation of Keratan Sulfate Proteoglycan—The 4 m guanidine
HCl extracts of corneal stromas were dialyzed into 8 m urea, and applied to a column of DEAE-cellulose to separate glycoproteins, which pass through or elute early in the salt
gradient, from the proteoglycans, which elute at higher salt
concentrations. A large 3H-labeled peak eluted in the unbound fraction along with a large peak of absorbance at 280 nm (Fig.

1The abbreviation used is: ConA, concanavalin A.
1. Additional peaks with \(^{3}H\) activity eluted near fraction 30 at the beginning of the salt gradient and between fractions 40 and 60 where the majority of \(^{35}S\) activity also eluted. The final 3.0 M NaCl wash eluted only a small peak near the salt gradient and between fractions 40–60, indicating that the salt gradient eluted the majority of the proteoglycans. The \(^{35}S\) and \(^{3}H\)-labeled peak in fractions 40–60 was pooled as shown by the bar in Fig. 1 to give the proteoglycan fraction. About 30% of the total \(^{3}H\) activity was recovered in the proteoglycan fraction while the rest was presumably in glycoproteins which did not bind to the column or which bound only weakly.

The DEAE-purified proteoglycan fraction was then incubated with chondroitinase ABC in the presence of protease inhibitors (27) to degrade the chondroitin-dermatan sulfate, dialyzed into urea, and applied to a column of DEAE-cellulose. The core protein molecules of the dermatan sulfate proteoglycans eluted in the unbound fractions as indicated by the \(^{3}H\) activity and absorbance at 280 nm in fractions 5–25 (Fig. 2). The intact keratan sulfate proteoglycans were eluted by the salt gradient between fractions 50 and 90 as shown by the peak of \(^{3}H\) and \(^{35}S\) activity and by the absorbance at 280 nm in this fraction. About 52% of the \(^{3}H\) activity was present in the keratan sulfate proteoglycan peak. Previously (20), it was shown that the macromolecular \(^{3}H\) activity incorporated with [\(\text{[2-}^{3}H\text{]}\)mannose as a precursor remains almost entirely in mannos and fucose.

The keratan sulfate proteoglycan fraction was recovered and eluted on a column of ConA-Sepharose (Fig. 3). Almost all of the \(^{3}H\) activity and 80% of the \(^{35}S\) activity bound to the column, eluting as a single peak in the presence of \(\alpha\)-methylmannoside. The column was then washed with another aliquot of 1 M \(\alpha\)-methylmannoside after 18 h (Fig. 3, arrow at fraction 40) to check for complete elution of the bound proteoglycans (29). Heparan sulfate proteoglycans, which eluted with the keratan sulfate proteoglycans on DEAE-cellulose, eluted in the unbound fractions on the concanavalin A column along with unbound keratan sulfate proteoglycan as described elsewhere (30). The ConA-bound material between fractions 22 and 35 in Fig. 3 was pooled, dialyzed against distilled water, and lyophilized, yielding ~50 mg of a highly purified keratan sulfate proteoglycan.

Isolation of the Linkage Region Oligosaccharides and the Mannose-containing Oligosaccharides—The purified keratan sulfate proteoglycan fraction was digested with papain and applied to DEAE-Sepharose. A mannose-containing oligosaccharide-peptide fraction eluted in the unbound fraction as shown by \(^{3}H\) activity between fractions 2 and 20 (Fig. 4). The keratan sulfate peptide fraction eluted in the latter half of the NaCl gradient as shown by both the \(^{35}S\) and \(^{3}H\) activity after fraction 50. The presence of a significant level of \(^{35}S\) activity at the end of the salt gradient (fraction 75) indicated that some keratan sulfate peptides had not eluted with 0.7 M NaCl, and therefore the column was washed further with 3.0

The purity of a similarly prepared proteoglycan fraction was assessed by polyacrylamide gel electrophoresis before and after digestion with chondroitinase ABC or endo-\(\beta\)-galactosidase. Coomassie blue staining of the gels indicated that the proteoglycans were the only components detected in this fraction (K. Nakazawa, unpublished data).
Keratan Sulfate Proteoglycan Purification

Fig. 4. DEAE-Sephacel chromatography of the papain digest of the ConA-bound keratan sulfate proteoglycan fraction isolated from the ConA-Sepharose column as indicated in Fig. 3.

Fig. 5. DEAE-Sephacel chromatography of the endo-β-galactosidase digest of the keratan sulfate peptide fraction isolated from the DEAE-Sephacel column as indicated in Fig. 4.

Fig. 6. Bio-Gel P-10 chromatography of the linkage region glycopeptide fraction isolated from the DEAE-Sephacel column as indicated in Fig. 5.

Fig. 7. Bio-Gel P-10 chromatography of the oligosaccharide-peptide fraction isolated from the DEAE-Sephacel column as indicated in Fig. 4.

M NaCl. Fractions with 3H and 35S activity that eluted with both the salt gradient and the 3.0 M NaCl wash were pooled as shown in Fig. 4 to give the keratan sulfate peptide fraction. About 39% of the 3H activity in the original purified keratan sulfate proteoglycan was recovered in the glycopeptide fractions, with the remainder present in the keratan sulfate peptide fraction.

The keratan sulfate peptide fraction was digested with endo-β-galactosidase, and the digest was applied to a column of DEAE-Sephacel. All of the 3H activity eluted in the unbound fractions along with a slightly retarded 35S-labeled peak (Fig. 5). Most of the 35S activity eluted as several peaks during the salt gradient and in the 3.0 M NaCl wash. These are keratan sulfate fragments of different sizes and charge densities (28).

The 3H peak in the unbound fractions along with the associated 35S peak was pooled as shown in Fig. 5 and lyophilized. The sample was then dissolved in a small volume of 0.4 M ammonium acetate, pH 5.5, and applied to a column of Bio-Gel P-10 (Fig. 6). The 35S activity eluted with a hexose-positive peak at the total column volume. This peak probably contains monosulfated disaccharides (28). A broad, 3H-labeled peak eluted with a hexose-positive peak in the middle of the column. This peak was pooled as shown in Fig. 6 to give the keratan sulfate linkage region glycopeptides. This fraction was lyophilized, yielding 2.4 mg. Its structure was determined as indicated in the accompanying paper (22).

The oligosaccharide-peptide fraction (Fig. 4) was isolated and applied to the Bio-Gel P-10 column (Fig. 7). Two broad peaks of 3H activity eluted within the excluded and partially included column volume. The middle-eluting peak II (between fractions 55 and 69) was accompanied by a substantial amount of hexose, and its elution position was almost the same as that of the keratan sulfate linkage region glycopeptides in Fig. 6. The large hexose peak eluting after fraction 70 is probably derived from α-methylmannoside, which was used to elute the keratan sulfate proteoglycan from the ConA-Sepharose column and which was incompletely dialyzed. The two 3H-labeled peaks were pooled as shown in Fig. 7 and lyophilized. The second (peak II) contained 6.5 mg. The structures of the oligosaccharides in this fraction were determined as described in the accompanying paper (22).

DISCUSSION

[2-3H]Mannose was used as a labeling precursor along with [35S]sulfate to label the keratan sulfate proteoglycan in explant cultures of monkey cornea (20). These labeled macromolecules were mixed with a large quantity of unlabeled macromolecules, and, by following the incorporated radioactivity, about 50 mg of highly purified keratan sulfate proteoglycan sample was isolated from 315 corneas by a series of steps including: first, DEAE chromatography, to isolate proteogly-
cans; second, chondroitinase ABC digestion followed by DEAE chromatography to remove the dermatan sulfate proteoglycan; and third, chromatography on immobilized concanavalin A to remove heparan sulfate proteoglycans (30). Of the total incorporated $^3$H activity, 15% was recovered in the purified keratan sulfate proteoglycan fraction (Fig. 2), 14% in the dermatan sulfate proteoglycan core protein fraction (Fig. 2), and 66% in the glycoprotein fraction (Fig. 1), respectively. Modifications of the procedures developed in this study should allow the purification of sufficient quantities of the oligosaccharide-peptides on the keratan sulfate proteoglycans to compare their structures with those present on the keratan sulfate proteoglycan (22).

In separate studies (30), procedures similar to those described above were used to show that both the dermatan sulfate proteoglycans and the keratan sulfate proteoglycans contain subpopulations based upon differences in oligosaccharide content and in numbers of glycosaminoglycan chains. In these studies, it was found that the concanavalin A step was essential for removing a small amount of heparan sulfate proteoglycan from the keratan sulfate proteoglycan fraction. However, a proportion (20–30%) of the keratan sulfate proteoglycan does not bind to the column, and appears to have fewer intact oligosaccharide-peptides (30).

After papain digestion of the keratan sulfate proteoglycan, glycopeptides, which contained 59% of the $^3$H activity, were separated from keratan sulfate peptides, which contained 41% of the $^3$H activity and all of the $^{35}$S activity. The ratio of $^3$H activity in these two fractions in combination with their structures (22) suggests that there is an average of approximately one intact oligosaccharide for each keratan sulfate linkage site in the intact proteoglycans.

The keratan sulfate peptides were treated with endo-$\beta$-galactosidase, an enzyme from Pseudomonas which selectively degrades keratan sulfate (27, 28). After digestion, the linkage region oligosaccharide-peptide fraction, which contained all of the $^3$H activity but no $^{35}$S activity, was purified. The absence of $^{35}$S activity indicates the effectiveness of the endo-$\beta$-galactosidase for removing all of the backbone structure of the chains from the linkage region. Clearly, then, the use of $[2-^3H]$mannose and $[^35S]$sulfate as labeling precursors facilitated the purification of the intact glycopeptides and the linkage region oligosaccharide-peptides in sufficient quantities for structural determination (22) while utilizing minimal amounts of material.

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