

Fractionation of Keratan Sulfate from Human Nucleus Pulposus

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Procedures are described for the fractionation of keratan sulfate from nucleus pulposus. By determination of the glucosamine to galactosamine ratio the average chain length could be assessed. These procedures, which fractionate keratan sulfate according to molecular size can be applied to the macro- as well as the microscale.

Keratan sulfate is composed of approximately equimolar proportions of galactose, *N*-acetyl glucosamine, and sulfate, and has been isolated from cornea,¹ nucleus pulposus,² and cartilage.³⁻⁵ The polymer generally exhibits a considerable degree of heterogeneity. In addition to the components of the repeating disaccharide unit, galactose and glucosamine, small amounts of sialic acid, fucose, and galactosamine are present.^{2,5-8} Whereas the galactosamine appears to be involved in *O*-glycosidic linkages to seryl and threonyl residues forming the carbohydrate-protein link of skeletal keratan sulfate-protein complex,⁸ the exact location of the sialic acid and fucose residues is not known.

Another type of heterogeneity consistently displayed by keratan sulfate is molecular weight polydispersity.⁹⁻¹¹ Furthermore, keratan sulfate shows a marked variation in the degree of sulfation^{4,5,7,11-13} as well as in the position of the sulfate groups.^{14,15}

In order to study the possible biological implications of this microheterogeneity, methods for the demonstration of structural variability are needed. The microcolumn technique developed in this laboratory¹⁶ has been successfully employed for analysis of molecular weight polydispersity of chondroitin sulfate.¹⁷ The present paper describes procedures, applicable to the microgram level, which permit fractionation of keratan sulfate according to molecular size.

EXPERIMENTAL

Materials. The nuclei pulposi were obtained from non-pathological autopsy cases and were dissected free from annulus fibrosus, dried in acetone, and ground in a Wiley mill.

Crystalline papain was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A., ECTEOLA-cellulose (=capacity 0.62 mequiv./g; batch 20066) was a product of Serva Entwicklungslabor, Heidelberg, Germany. Cetylpyridinium chloride (CPC) was purchased from AB Recip, Stockholm, Sweden.

Analytical methods. Hexosamine was determined by the Elson and Morgan procedure¹⁸ after hydrolysis with 6 M HCl for 8 h in a boiling water bath and subsequent removal of HCl in a desiccator over NaOH pellets under reduced pressure.¹⁶ Separation of glucosamine and galactosamine was performed according to the method of Antonopoulos.¹⁹ Hexoses were estimated by the anthrone method as modified by Goa,²⁰ and sulfate by the benzidine method of Antonopoulos.²¹

Ion exchange chromatography of keratan sulfate fractions was carried out on columns (0.3 × 6 cm) of ECTEOLA-cellulose (Cl⁻). The columns were eluted with 1 ml of water, 0.02 M HCl, 0.8 and 1.0 M NaCl, and finally with 6 M HCl. The NaCl effluents were diluted five times with water and adsorbed on columns (0.3 × 6 cm) of ECTEOLA-cellulose which were washed with water and eluted with 6 M HCl. The various HCl fractions were then hydrolysed and analysed for glucosamine and galactosamine as described above.

Keratan sulfate samples (5 mg) were chromatographed on columns (1.1 × 185 cm) of Sephadex G-150, fine, eluted with 0.25 M NaCl—0.02 % NaN₃. Fractions (2 ml) were collected at a rate of 5 ml/h. One ml aliquots were hydrolysed and analysed for hexosamine as described above.

Isolation and fractionation of keratan sulfate. In a typical experiment 1 g of tissue powder was digested with papain as described by Scott.²² The digest was fractionated on a column (4 × 80 cm) of cellulose equilibrated with 1 % aqueous cetylpyridinium chloride (CPC). The column was eluted with 1000 ml of 1 % CPC, 0.30 M NaCl—0.05 % CPC, 0.65 M MgCl₂—0.05 % CPC, 0.05 % CPC, and finally propanol-1 % CPC, 1:1 (v/v). The 1 % CPC fraction (I, Fig. 1) was treated with 5 volumes of ethanol and 10 ml of 25 % (w/v) aqueous sodium acetate. The precipitate was collected by centrifugation, washed with absolute ethanol, ether, and air dried. The yield was 120 mg. The propanol-1 % CPC fraction (IV, Fig. 1) was clarified with small amounts of propanol and then precipitated with ethanol-sodium acetate and processed as above. The yield was 3 mg.

The material eluted with 1 % CPC was further fractionated as follows. 100 mg was dissolved in 5–10 ml of water and excess 1 % CPC was added (until a clear solution was obtained). By the addition of NaCl to a final concentration of 0.4 M a precipitate was obtained. After centrifugation, the supernatant solution (II, Fig. 1) was treated with 5 volumes of ethanol and dried polysaccharide was obtained as described above. The yield was 32 mg. The material precipitated at 0.4 M NaCl (III, Fig. 1) was dissolved in 5 ml of propanol-1 % CPC, 1:1 (v/v) and reprecipitated with ethanol containing sodium acetate as above. The yield was 55 mg.

RESULTS AND DISCUSSION

Fractionation of keratan sulfate from human nucleus pulposus yielded one major (Fraction I, Fig. 1) and one minor component (Fraction IV, Fig. 1) when the tissue digest was chromatographed on a cellulose column saturated with CPC.²³ The material eluted with 1 % CPC was further fractionated by the addition of salt into one soluble and one insoluble component (Fractions II and III, Fig. 1). The analytical data for these fractions are shown in Table 1. Whereas fraction I contained 16 % of total hexosamine as galactosamine, fraction IV contained only 2 %. The two subfractions of fraction I, *i.e.* II and III, also differed with respect to their galactosamine content, 21 and 11 %, respectively.

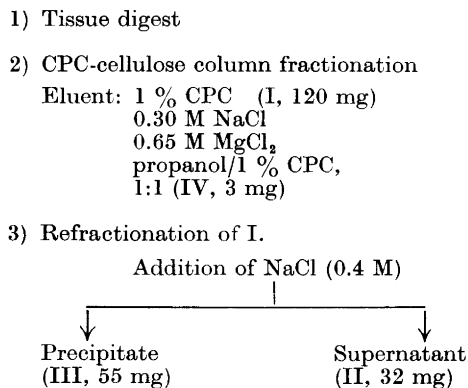


Fig. 1. Flow diagram of the various keratan sulfate fractions from human nucleus pulposus.

Table 1. Analytical data of various keratan sulfate fractions.

Frac- tion	Hexosamine	Galactose	Sulphate	Glucosamine	Galactosamine
	% of dry weight	% of dry weight	% of dry weight	% of total hexosamine	% of total hexosamine
I	25.0	24	16.1	84	16
II	25.2	25	14.0	79	21
III	26.0	27	17.0	89	11
IV	23.0	24	14.5	98	2

respectively. On the other hand, all four fractions showed essentially similar molar ratios of sulfate to hexosamine. Since skeletal keratan sulfate appears to be linked to protein by way of an *O*-glycosidic bond between a galactosamine moiety and seryl or threonyl residues in the polypeptide,⁸ the ratio between glucosamine and galactosamine would be a measure of the polysaccharide chain length provided the polysaccharide contains one galactosamine residue per chain. Therefore the fractions described here which displayed variable galactosamine content were subjected to gel chromatography in order to assess their relative molecular size. As shown in Fig. 2, fraction IV was eluted near the void volume, fraction III was more retarded, and fraction II was the most retarded, but displayed an elution pattern indicative of considerable heterogeneity. Thus, the results of the gel chromatography suggested that molecular size was inversely proportional to the galactosamine content.

The keratan sulfate fraction which was eluted with 1 % CPC (Fraction I, Fig. 1) was also subjected to chromatography on ECTEOLA-cellulose. The results are shown in Table 2. It is evident that this technique also has the

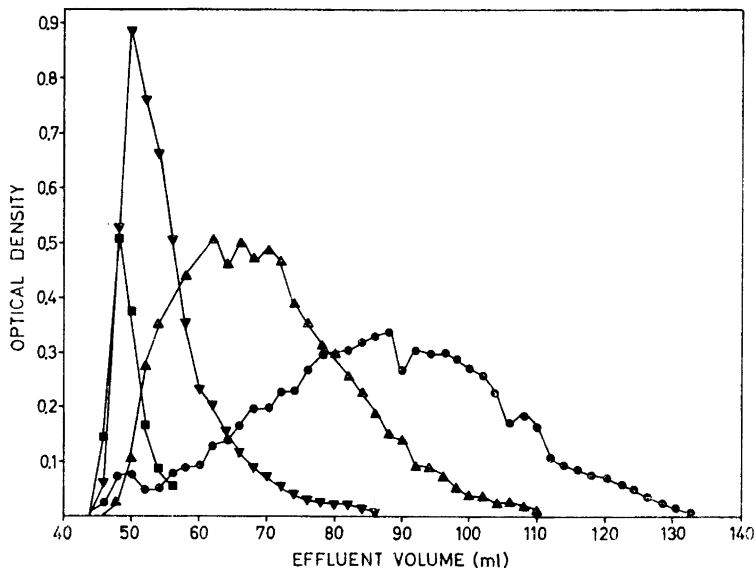


Fig. 2. Gel chromatography of various keratan sulfate samples on a column calibrated with blue dextran.

■, Blue dextran;
▲, Fraction III;

▼, Fraction IV (Table 1);
●, Fraction II.

Table 2. Analyses of keratan sulfate fractions obtained by ECTEOLA column chromatography of fraction I (Table 1).

Eluent	% of total hexosamine	Glucosamine	Galactosamine
		% of total hexosamine in the fraction	
0.02 M HCl	0	—	—
0.8 M NaCl	23	73	27
1.0 M NaCl	36	86	14
6.0 M HCl	42	92	8

potentials of fractionating keratan sulfate according to molecular size; the galactosamine content varied between 8 and 27 % of total hexosamine. These results are thus in agreement with those of Laurent and Scott¹⁰ who showed that corneal keratan sulfate may be fractionated on ECTEOLA-cellulose according to molecular weight.

The results presented here indicate several possibilities for fractionation of keratan sulfate according to molecular size applicable to the macroscale

as well as to the microscale. Treatment of keratan sulfate with CPC in the presence of salt leads to a selective precipitation of the larger molecules. This technique, which has not been described earlier, is particularly suitable for macroprocedures. However, analysis of small samples may be performed in the following way. Tissue digests are applied to CPC-cellulose columns¹⁶ and the 1 % CPC effluent is extracted with isoamylalcohol in order to remove CPC and the aqueous phase is subsequently applied to an ECTEOLA column for further fractionation. In this way the water and 0.02 M HCl effluents from the ECTEOLA column will contain glycopeptides, while the keratan sulfate is eluted later. Details concerning this microprocedure will be presented elsewhere.

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REFERENCES

1. Meyer, K., Linker, A., Davidson, E. A. and Weissmann, B. *J. Biol. Chem.* **205** (1953) 611.
2. Gardell, S. and Rastgeldi, S. *Acta Chem. Scand.* **8** (1954) 362.
3. Meyer, K., Hoffman, P. and Linker, A. *Science* **128** (1958) 896.
4. Furuhashi, T. *J. Biochem. Tokyo* **50** (1961) 546.
5. Mathews, M. B. and Cifonelli, J. A. *J. Biol. Chem.* **240** (1965) 4140.
6. Gregory, J. D. and Rodén, L. *Biochem. Biophys. Res. Commun.* **5** (1961) 430.
7. Seno, N., Meyer, K., Anderson, B. and Hoffman, P. *J. Biol. Chem.* **240** (1965) 1005.
8. Bray, B. A., Liebermann, R. and Meyer, K. *J. Biol. Chem.* **242** (1967) 3373.
9. Laurent, T. C. and Anseth, A. *Exptl. Eye Res.* **1** (1961) 99.
10. Laurent, T. C. and Scott, J. E. *Nature* **202** (1964) 661.
11. Greiling, H. and Stuhlsatz, H. W. *Z. Physiol. Chem.* **345** (1966) 236.
12. Cifonelli, J. A., Saunders, A. and Gross, J. I. *Carbohydr. Res.* **3** (1967) 478.
13. Fransson, L.-Å. and Anseth, A. *Exptl. Eye Res.* **6** (1967) 107.
14. Bhavanandan, V. P. and Meyer, K. *J. Biol. Chem.* **242** (1967) 4352.
15. Bhavanandan, V. P. and Meyer, K. *J. Biol. Chem.* **243** (1968) 1052.
16. Antonopoulos, C. A., Gardell, S., Szirmai, J. A. and de Tyssonsk, E. R. *Biochim. Biophys. Acta* **83** (1964) 1.
17. Szirmai, J. A., de Tyssonsk, E. van B. and Gardell, S. *Biochim. Biophys. Acta* **136** (1967) 331.
18. Elson, L. A. and Morgan, W. T. J. *Biochem. J.* **27** (1933) 1824.
19. Antonopoulos, C. A. *Arkiv Kemi* **25** (1966) 243.
20. Goa, J. *Scand. J. Clin. Lab. Invest., Suppl.* **22** (1955) 7.
21. Antonopoulos, C. A. *Acta Chem. Scand.* **16** (1962) 1521.
22. Scott, J. E. *Methods Biochem. Anal.* **8** (1960) 145.
23. Antonopoulos, C. A. *Acta Univ. Lund* **2** (1965) 35.

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