A Simple and Rapid Method for Isolation of Reduced Carbohydrate Fragments from the Linkage Region of Cartilage Keratan Sulphate

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The method described is a simple and rapid procedure for isolation in high yield of carbohydrate fragments containing terminal galactosaminitol derived from the polysaccharide–protein linkage region of cartilage keratan sulphate. It is based on the observation that reducing sugars bind tightly to Dowex-1 resin (hydroxide form), whereas reduced analogues (sugar alcohols) do not [H. Yamaguchi, S. Inamura & K. Makino (1976) *J. Biochem. (Tokyo)* 79, 299–303].

Cartilage proteoglycans often contain the glycosaminoglycan keratan sulphate (Gregory & Rodén, 1961), which is covalently attached to the protein component of the proteoglycan by *O*-glycosidic linkages between *N*-acetylgalactosamine in the polysaccharide and threonine and serine residues in the protein (Bray *et al.*, 1967). In the course of studies on the structure of cartilage keratan sulphate the method described in the present paper was devised for isolation of reduced carbohydrate fragments from the linkage region.

Materials and Methods

The keratan sulphate used in the present study was prepared from bovine nasal septum by a method, previously described (Kieras, 1974), that involved papain digestion of the tissue, ethanol fractionation for enrichment of keratan sulphate, and purification on Dowex-1 (chloride form). The material eluted with 2M-NaCl was used and had the following percentage dry weight composition: hexose, 24.8% (1.38 $\mu\text{mol/mg}$); hexosamine, 21.2% (1.19 $\mu\text{mol/mg}$); sialic acid, 6.3% (0.20 $\mu\text{mol/mg}$); and uronic acid, 1.3% (0.067 $\mu\text{mol/mg}$). The sulphate/hexosamine ratio was 1.06:1. A Technicon TSM amino acid analyser showed the material to contain 90% glucosamine (0.997 $\mu\text{mol/mg}$) and 10% galactosamine (0.112 $\mu\text{mol/mg}$) as the only amino sugars. Amino acids accounted for 7.6% of the material. The methods used for analysis of the various sugar components have been described (Kieras, 1974).

β -Elimination and reduction of keratan sulphate peptide was carried out at a concentration of 10 mg/ml in 0.05M-NaOH, 1M-NaBH₄ at 50°C for 23 h as previously described (Kieras, 1975). The reaction was terminated in an ice bath by addition of acetic acid to a final pH of 4.5 in the presence of a few drops of octanol to prevent foaming. The preparation was then dialysed against water to remove salts, concentrated, and analysed for various sugar components.

After this treatment, 80% of the hexose and 90% of the glucosamine was recovered; 80% of the original galactosamine was destroyed by the β -elimination and reduction and 79% of that destroyed was converted into galactosaminitol, in agreement with previous results (Kieras, 1975).

Mild acid hydrolysis of reduced keratan sulphate was carried out at a concentration of 1% (w/v) or less in 0.2M-trifluoroacetic acid at 100°C for 3 h in sealed tubes. Trifluoroacetic acid was removed by repeated evaporation with water under vacuum at 40°C. The residues were redissolved in water and analysed for sugar components. Hydrolysis for amino sugar determination was carried out in 4M-HCl for 8 h at 100°C, and hydrolysates were treated as described above to remove acid.

The separation of reduced fragments from reducing fragments was performed with Dowex-1 (X8; 200–400 mesh; hydroxide form; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Immediately before use the resin was treated sequentially with 2M-HCl, water, 2M-NaOH and water to convert it into the hydroxide form. For 100 mg of starting keratan sulphate a column containing 10 ml of resin was used. The sample was applied and allowed to enter the resin, the flow interrupted for 10 min, then the resin was eluted with 10 bed volumes of water, and finally with 10 bed volumes of 1M-HCl or 1M-NH₃. The eluates were evaporated to dryness, redissolved in water, and analysed for various sugars.

Reduced fragments from the water eluate of the Dowex-1 column were analysed by paper chromatography as previously described (Kieras, 1974). G.l.c. was also used for analysis of monosaccharides and disaccharides in the mixture. The samples were *N*-acetylated with acetic anhydride, and trimethylsilyl derivatives were prepared (Clamp *et al.*, 1971). Analyses of the trimethylsilyl derivatives were performed by using glass columns of internal diameter 4 mm and 1.83 m (6 ft) in length, containing 3% OV-17 on Supelcoport (80–100 mesh; Supelco Co.,

Bellefonte, PA, U.S.A.). N₂ was used as carrier gas at a flow rate of 45 ml/min, and the column was operated at 170°C for monosaccharides and 250°C for disaccharide analyses. All analyses were carried out with a Packard 417 gas-liquid chromatograph equipped with flame-ionization detectors.

The disaccharide *N*-acetylgalactosaminyl- α -(1 $\rightarrow$ 3)-*N*-acetylgalactosaminitol [GalNAc- α -(1 $\rightarrow$ 3)-GalNAcH₂] was a gift from Dr. E. A. Kabat of Columbia University College of Physicians and Surgeons, New York, NY, U.S.A. Galactosyl-(1 $\rightarrow$ 3)-*N*-acetylgalactosaminitol [Gal-(1 $\rightarrow$ 3)-GalNAcH₂] was prepared as previously described (Kieras, 1974). All chemicals used were reagent grade, and other sugars were obtained from commercial sources.

Results and Discussion

In previous work (Kieras, 1974) reduced oligosaccharides were prepared by β -elimination and reduction of glycopeptides produced by mild acid hydrolysis of keratan sulphate. In the present procedure the keratan sulphate is β -eliminated and reduced, and, after mild acid hydrolysis, the reduced fragments are isolated by chromatography on Dowex-1 ion-exchange resin.

Complete hydrolysis of reduced keratan sulphate to monosaccharides was carried out under the conditions

used for amino sugar analysis (4M-HCl, 8h, 100°C). After removal of acid, the hydrolysate was applied to a Dowex-1 column and eluted with water. The only sugar found in the water eluate is galactosaminitol (85% yield); no hexose, hexosamine or reducing sugar was found in the water eluate, indicating that all of the reducing sugar had been retained by the resin (Table 1).

In the following experiment the reduced keratan sulphate was hydrolysed to a mixture of monosaccharides and oligosaccharides in 0.2M-trifluoroacetic acid at 100°C for 3h; similar results were obtained if the mild acid hydrolysis was performed with 0.1M-HCl at 100°C for 3h (results not shown). It was assumed that hydrolysis of reduced keratan sulphate would produce fragments similar to those obtained by hydrolysis of peptide-bound keratan sulphate. After removal of the acid, the hydrolysate was applied to the Dowex-1 column and eluted with water. In this case 72% of the original galactosaminitol was recovered in the water eluate, and, in addition, hexose, glucosamine and galactosamine were also found in this fraction (Table 1). These sugars were bound in oligosaccharides terminated by galactosaminitol and comprised about 50% of the total sugar in the water eluate. The components of this fraction were analysed by paper chromatography and shown to be free *N*-acetylgalactosaminitol, the

Table 1. *Sugar analyses of fractions derived from reduced keratan sulphate by acid hydrolysis and Dowex-1 treatment* Analyses were carried out as described in the Materials and Methods section. Numbers in parentheses are percentage recoveries based on reduced keratan sulphate, except where noted.

Material and treatment	Total amount (μ mol)				
	Hexose as galactose	Glucosamine	Galactosamine	Galactosaminitol	Reducing sugar as galactose
Reduced keratan sulphate*	108.3	88.2	2.1	7.2	0
Reduced keratan sulphate; 4M-HCl, 8h, 100°C, Dowex-1, water eluate	0	0	0	6.1 (85)	0†
Reduced keratan sulphate; 0.2M-trifluoroacetic acid, 100°C, 3h, Dowex-1, water eluate	6.7 (6.2)	0.8 (0.9)	0.2 (9.5)	5.2 (72)	0.6 (0.4)‡
Reduced keratan sulphate; 0.2M-trifluoroacetic acid, 100°C, 3h, Dowex-1, 1M-HCl eluate, after elution with water	1.0 (0.9)	0.4 (0.5)	0	1.3 (18)	0.2 (0.1)‡

* Derived from 100mg of keratan sulphate. For analyses of starting material and recoveries after β -elimination and reduction, see the Materials and Methods section.

† Before treatment with Dowex-1, reducing sugar as galactose was 154 μ mol.

‡ These percentage recoveries were calculated on the basis of reducing sugar in the complete acid hydrolysate (154 μ mol as galactose).

disaccharide Gal-(1→3)-GalNAcH₂ and larger fragments terminated by galactosaminitol, in agreement with previous work (Kieras, 1974). These results justified the assumption that mild acid hydrolysis of reduced keratan sulphate produced fragments similar to those obtained by hydrolysis of keratan sulphate peptide. The components of the water fraction were also analysed by g.l.c., and the results were in agreement with those obtained by paper chromatography. I decided to find out whether the disaccharide GalNAc- α -(1→3)-GalNAcH₂ was present in the mixture, since this fragment has been isolated from blood-group substances (Maisonrouge-McAuliffe & Kabat, 1976) and because small amounts of galactosamine were found in the water eluate. It was, however, not possible to detect this component in the mixture.

The incomplete recovery of galactosaminitol from the complete acid hydrolysate (85% in Table 1) is not understood, although in other experiments (results not shown) yields of 90% or greater have been obtained. The lower yield of galactosaminitol from the mild acid hydrolysate (trifluoroacetic acid) (72% in Table 1) is most likely due to the presence of sulphated and reduced oligosaccharides that bind to the Dowex-1 and are not eluted with water. It is known that hydrolysis of keratan sulphate under the conditions used here for mild acid hydrolysis does not cause complete desulphation (F. J. Kieras, unpublished work). The succeeding elution with 1M-HCl releases an additional 18% of the galactosaminitol (Table 1) and small amounts of hexose and glucosamine. This represents an overall recovery of 90% of the galactosaminitol applied to the column. Elution of the Dowex-1 with 1M-HCl (or 1M-NH₃; results not shown) did not release large amounts of bound reducing sugar, indicating that these fragments containing galactosaminitol are bound by sulphate groups. If only neutral oligosaccharides are desired, the entire procedure can be applied to desulphated keratan sulphate (Choi & Meyer, 1975).

The results of these experiments clearly demonstrate that the method of Yamaguchi *et al.* (1976) can be used to isolate the reduced linkage-region oligosaccharides from complex heteropolysaccharides

such as keratan sulphate. Analysis of these fragments shows them to be quite similar in size and composition to those prepared by more laborious methods (Kieras, 1974). Acidic fragments containing sulphate (or uronic acids) can be removed from the resin by elution with an appropriate solvent (HCl or NH₃). The method is applicable to neutral, acidic or basic homo- or hetero-polysaccharides and may prove useful in isolation of oligosaccharides from glycoproteins. Application of this procedure to such complex molecules as proteoglycan subunit from bovine nasal septum containing only 7% keratan sulphate (Hascall & Sajdera, 1970) has allowed isolation of oligosaccharides derived from the linkage region of keratan sulphate. The yield was approximately 70% of the galactosamine attributable to the linkage region of keratan sulphate, and the presence of the disaccharide Gal-(1→3)-GalNAcH₂ was also demonstrated (F. J. Kieras, unpublished work).

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