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Carbohydrate Components in the Epithelial Mucin of Hagfish, *Myxine glutinosa*

A. LEHTONEN, J. KÄRKKÄINEN and E. HAAHTI

Department of Medical Chemistry, University of Turku, Turku 3, Finland

The sulphate-containing heteropolysaccharides of hagfish (*Myxine*) mucin were isolated and fractionated by elution of CPC-precipitated * polysaccharide on a cellulose column. Electrophoretically the polysaccharide could be divided into three major fractions.

The sulphate content of the fractions varied between 8 and 37 %. The monosaccharide components (galactose, fucose, mannose, glucosamine, and galactosamine) of the fractions were determined by gas chromatography. Qualitative and quantitative information is presented.

Epithelial mucins belong to the group of "neutral" mucosubstances [1] containing varying proportions of carbohydrate (consisting usually of amino sugars, galactose, mannose, fucose and sialic acids) combined with protein.[2] Ester sulphate is present in the intestinal mucin of sheep and guinea pig.[3] Inoue [4] has isolated a sulphated heteropolysaccharide named horatinsulphuric acid from a gastropod, *Charonia lampas*.

This report deals with the carbohydrates of the dermal mucin secreted by a cyclostome, *Myxine*. The components were analyzed quantitatively by gas chromatography, taking advantage of the recent developments in the use of trimethylsilyl derivatives for the analysis of monosaccharides.[5–12]

MATERIALS AND METHODS

Material. Epithelial mucus of *Myxine* (33 g) was stored in the frozen state. The sample was hydrolyzed with papain as described by Schiller *et al.*[13] Trichloroacetic acid (10 %, w/v) was added to precipitate residual proteins and nucleic acids, and the excess TCA was removed from the supernatant by extraction with ether.

The mucopolysaccharides were precipitated at +4° with 96 % ethanol (four volumes), containing 0.5 % of sodium acetate, and the precipitate was dissolved in water. Only a trace of hexosamine was left in the supernatant.

* Abbreviations: CPC, cetyl pyridinium chloride; TMS, trimethylsilyl-; TCA, trichloroacetic acid.

The column fractionation of mucopolysaccharides was performed on cellulose columns impregnated with a 1 % aqueous solution of cetyl pyridinium chloride (CPC; Recip AB., Sweden) as described by Antonopoulos *et al.*[14] Elution of mucopolysaccharides was carried out both stepwise and by applying a gradient. Stepwise elution: column 3 × 30 cm, sample about 65 mg, eluents 1 % CPC, 0.1, 0.25, 0.5, 1.0, 1.25, 1.5, 2.0, and 3.0 N $MgCl_2$. Gradient elution: column 1 × 10 cm, sample 5 mg, continuous gradient from water to 2.3 N $MgCl_2$. The polysaccharide fractions were reprecipitated at +4° with ethanol and the precipitates were dissolved in water.

The approximate molecular weight of mucopolysaccharides was determined using a Sephadex G-25 (Pharmacia AB, Uppsala, Sweden) column (1 × 35 cm) with blue dextran (molecular weight 2 000 000) as the reference compound.

Electrophoresis of the polysaccharide fractions was performed on "Oxoid" cellulose-acetate sheets, using a barbiturate buffer, pH 8.6, voltage gradient 10 V/cm, and a running time of 25 min.[20] The strips were stained with alcian blue (1 % solution w/v, in 25 % acetic acid w/v).

Quantitative colorimetric analyses. The uronic acids were analyzed by a modified carbazole reaction.[15] Amino sugars were liberated from the polysaccharides by hydrolysis for 17 h in 1 N HCl in sealed tubes at +103°, and analyzed by the Elson-Morgan reaction according to the modification of Boas.[16] A modified anthrone method [17] was used to estimate the neutral sugars. Sulphate was determined by the benzidine method.[18] Sialic acid was determined by the thiobarbituric acid reaction according to Aminoff.[19]

Gas-chromatographic methods. The fractions obtained from the cellulose column were hydrolyzed in 1 N hydrochloric acid at +103° for 17 h and hexosamines were separated from neutral sugars using columns of Dowex-50 according to Boas.[16] After mixing the eluates with the internal standard (sorbitol) the aqueous solutions were evaporated to dryness in a stream of nitrogen. Pyridine (0.14 ml), hexamethyldisilazane (0.04 ml) and trimethylchlorosilane (0.02 ml) were added successively, and the mixture was left to stand at room temperature for 30 min. The excess reagents were evaporated off in a stream of nitrogen; the trimethylsilyl (TMS-)-ethers were extracted with 5 ml of hexane and the solution was concentrated to the desired volume. Samples of the hexane solution (1−2 μl) were injected into the gas chromatograph with a 10 μl Hamilton-microsyringe.

A Barber Colman M-10 chromatograph equipped with a flame ionization detector was employed. The conditions in the column were as follows: 3 mm × 180 cm, 1 % SE-30 on 100−140 mesh siliconized Gas-chrom P (Applied Science Laboratories Inc., State College, Penn., U.S.A.), temperature + 140°, carrier gas, nitrogen, flow rate 30 ml/min, pressure 0.5 atm.

For *quantitative analyses* the peaks were separated and each component was weighed. The response of the flame ionization detector for each monosaccharide with sorbitol as reference was determined with standard compounds. Reproducibility of the peak areas (expressed as the relative standard deviation between duplications), $2d^2/(N-1)$ when d is the difference and N the number of duplicates was for glucosamine ±4.7 %, galactosamine ±6.4 %, galactose 4.4 %, mannose 1.5 %, and fucose 23.5 %, respectively. The values are calculated from 5 determinations from independently silylated samples.

Reagents. D-Glucosamine hydrochloride, homogeneous on paper chromatography, and D-galactosamine, homogeneous on paper chromatography, obtained from Mann Research Laboratories Inc., New York, N.Y., D(+)-glucose, analytical reagent grade (B.D.H., Poole, Great Britain) galactose, puriss. and L(−)-fucose, obtained from Fluka AG, Buchs SG, D-mannose "für die Mikroskopie und Bakteriologie" (E. Merck AG, Darmstadt) were used as references. Pyridine, reagent grade (J. T. Baker Chemical Co., Phillisburg, N. J.) hexamethyldisilazane, *purum* 98 % (Fluka AG, Buchs SG) and trimethylchlorosilane *puriss.* 99 % (Fluka AG) were used without further purification.

RESULTS

After the papain digestion of 33 g of fluid epithelial mucus, 232 mg of the polysaccharide material was precipitated with ethanol. The nitrogen content of the material was 10.1 % of the dry weight.

Fractionation of cetyl pyridinium chloride-precipitated carbohydrate on cellulose columns. The material obtained with ethanol precipitation was separated into fractions by eluting the CPC-precipitated mucopolysaccharides from cellulose columns with salt solutions containing stepwise increasing concentrations of $MgCl_2$. Analytical data for these fractions are shown in Table 1. The fractions eluted by 0.1 N $MgCl_2$ contained too little carbohydrate to make possible the quantitative determinations. Uronic acids were present only in significant amounts in the fraction which was not precipitated with

Table 1. Analytical data for polysaccharide fractions obtained by column chromatography on cellulose. The molar proportions are given in Fig. 4. The yield 73 %.

Eluent	Hexosamine mg	Hexose mg	Sialic acid mg	SO_4 mg	Nitrogen mg	Protein * mg
1 % CPC	2.06	6.50	0.61	0.85	1.77	9.8
0.25 N $MgCl_2$	0.37	0.96	0.38	0.08	0.20	0.9
0.50 N $MgCl_2$	0.63	1.44	0.06	0.24	0.28	1.4
1.00 N $MgCl_2$	1.67	2.60	1.14	1.12	0.52	2.1
1.25 N $MgCl_2$	1.16	2.03	0.10	1.08	0.50	2.6
1.50 N $MgCl_2$	1.58	2.02	0.35	1.32	0.86	4.5
2.00 N $MgCl_2$	3.46	4.19	0.55	4.05	1.06	4.8
3.00 N $MgCl_2$	1.80	2.75	0.13	2.70	0.80	4.1
Total	12.73	22.49	3.32	11.44	5.98	30.2

* Calculated from nitrogen which is present in excess of hexosamine and sialic acid, by multiplication by 6.25.

CPC and the molar ratio of hexosamines to uronic acids was 3.1:1. This fraction constituted about 15 % of the whole mucopolysaccharide material and contained 15.7 % protein nitrogen. Amino sugars and sulphate were present in all fractions. The fraction eluted by 0.25 N $MgCl_2$ contained least sulphate, the ratio of hexosamine to sulphate was 2.6:1. The sulphate content was highest in the fraction eluted with 3 N $MgCl_2$.

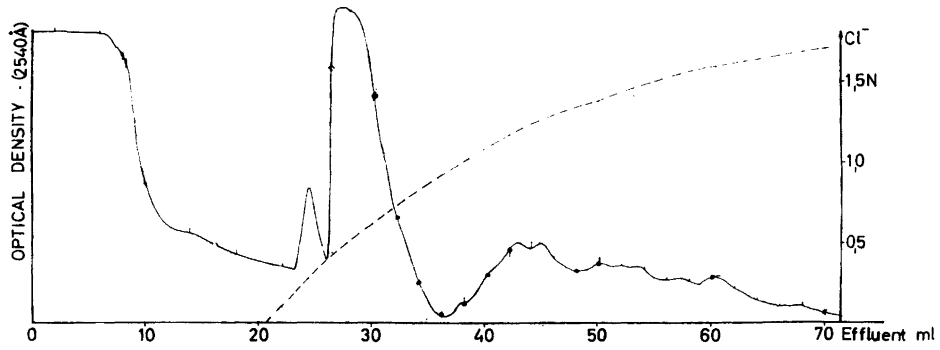

Fig. 1. Recording of the fractionation pattern of CPC-precipitated polysaccharide from *Myxine* dermal mucin. Ordinate, the absorption of released pyridine moiety, measured at 2540 Å. The actual concentrations of $MgCl_2$ are indicated.

Fractionation of CPC-precipitated polysaccharide by elution with a salt concentration gradient. The elution with continuous gradient is shown in Fig. 1. Two additional experiments gave similar results. This elution indicates that the polysaccharide is very heterogeneous and that the results based on stepwise elution are somewhat unreliable.

Determination of amino sugars and neutral sugars. Typical gas-chromatographic records are presented in Figs. 2—3. Both glucosamine and galactosamine were present in all the polysaccharide fractions. Galactose and fucose were present in all, and mannose in most fractions (Fig. 4).

Electrophoresis. Cellulose-acetate electrophoresis separated the fractions into three groups (Fig. 5). The electrophoretic mobility of the polysaccharides eluted with 0.1—0.25 N $MgCl_2$ corresponded to hyaluronic acid isolated from human umbilical cord. The fractions eluted with 0.5—2.0 N $MgCl_2$ moved

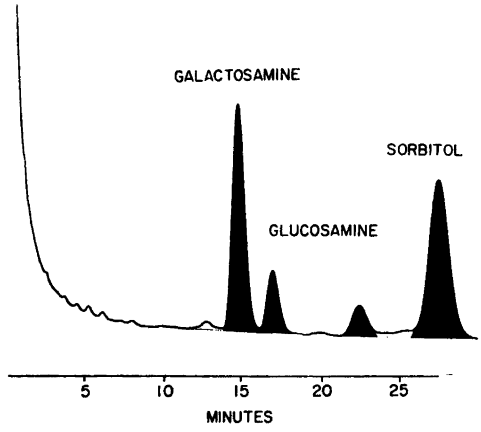

Fig. 2. Gas chromatographic pattern of hexosamines in a hydrolyzed polysaccharide fraction of *Myxine* mucin. (The 2.0 N $MgCl_2$-eluted fraction in Table 1).

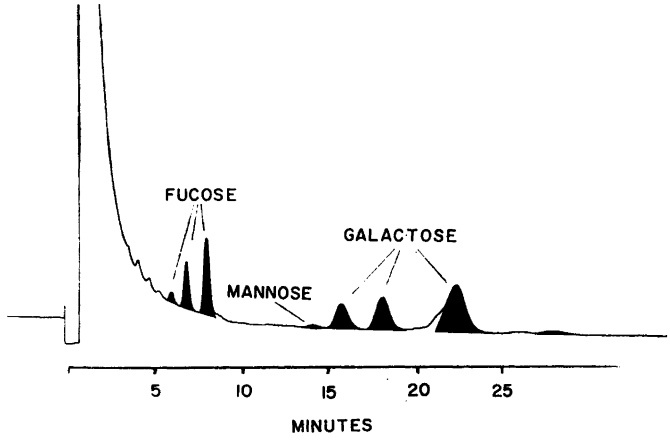

Fig. 3. Gas chromatographic pattern of neutral sugars in a hydrolyzed polysaccharide fraction in *Myxine* mucin. (The 0.25 N $MgCl_2$-eluted fraction in Table 1).

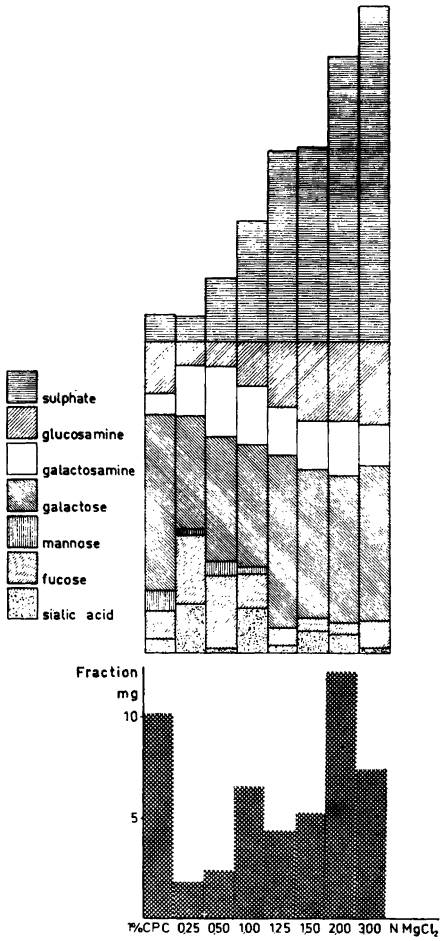

Fig. 4. The amount and the molar composition of the polysaccharide fractions obtained by elution with solutions containing stepwise increasing salt concentrations on the cellulose column (*cf.* Table 1).

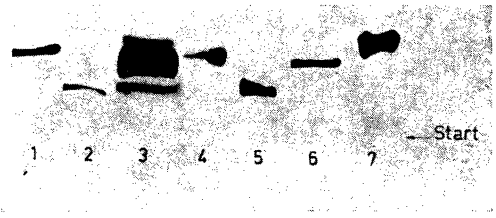

Fig. 5. Electrophoretic migration of polysaccharide fractions (Table 1, Fig. 3); *1* chondroitin sulphate; *2* hyaluronic acid; polysaccharides of *Myxine* mucin; *3* the unfractionated sample; *4* the fraction not precipitated with CPC; *5* 0.25 N $MgCl_2$-fraction; *6* 1.5 N $MgCl_2$-fraction; *7* 3.0 N $MgCl_2$-fraction.

with a mobility similar to that of chondroitin sulphate obtained from bovine skin. The electrophoretic mobility of the fraction not precipitated with CPC corresponded to chondroitin sulphate. It is believed that the low molecular weight of this fraction may be the reason for its solubility in CPC solution. The fraction obtained with 3 N $MgCl_2$ and containing most sulphate, moved farthest toward the anode.

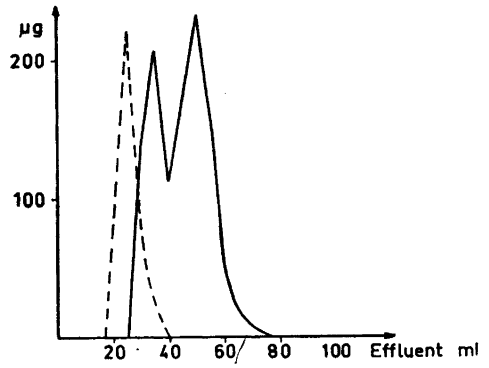

Fig. 6. Chromatography of the polysaccharides of *Myxine* mucin on Sephadex G-25 column. —— hexosamine by Elson-Morgan reaction; ------ absorbancy at 500 mµ ("blue dextran").

Molecular weight of the mucopolysaccharides. The mucopolysaccharides were eluted as two peaks behind that of blue dextran when gel-filtered through Sephadex G-25 column (Fig. 6). The results indicate that the molecular weight of both fractions is less than 5000.

DISCUSSION

The gas-chromatographic methods for the quantitative estimation of glucosamine and galactosamine, as also for the neutral sugars, are simple and reliable because the monosaccharides are separated before any results are calculated. Less than 1 µg of each monosaccharide can be quantitatively analyzed. Up until now quantitative work has largely been dependent on colorimetric methods, which in many cases are far from reliable, when mixtures of sugars are concerned.[21]

Because the simultaneous gas chromatography of hexoses and hexosamines usually leads to overlapping peaks, we separated the two classes of sugars using columns of Dowex-50. Sorbitol is very suitable as an internal standard because the TMS-derivative of sorbitol is well separated on SE-30 from those of all the other common hexoses and hexosamines. The use of calibration coefficients for the peak areas is necessary owing to the different response of the flame ionization detector for the individual TMS-sugars. Reproducibility of the peak areas, except that of fucose, was quite satisfactory and the amount of total hexosamines or neutral sugars compared well with the values obtained using Elson-Morgan and anthrone reactions, respectively.

The heterogeneity of hagfish mucin seems to arise mainly from the sulphate content and carbohydrate composition.

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