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Isolation and Characterization of the Glycosaminoglycans of Guinea Pig Rib Cartilage

STEFAN LOHMANDER,[a] ULF FRIBERG[a] and CHRISTOS A. ANTONOPOULOS[b]

[a] *Department of Histology, Karolinska Institutet, Fack, S-104 01, Stockholm 60, Sweden*
[b] *Department of Physiological Chemistry, University of Lund, Chemical Center, P.O.B. 740, S-220 07 Lund 7, Sweden*

The glycosaminoglycans of guinea pig costal cartilage were isolated by the CPC-cellulose column technique. The fractions were characterized with regard to content of hexosamine, hexuronic acid, hexose, sulfate, and susceptibility to digestion with testicular hyaluronidase. Infrared spectroscopy was also performed. Molecular size was determined by gel chromatography.

The total hexosamine content of costal cartilage was found to be 5.3 % of the dry weight. About 9 % thereof was derived from glycoproteins. Chondroitin sulfates accounted for about 80 %, and keratan sulfate for about 8 % of the total hexosamine content. The remaining 3 % presumably corresponded to hyaluronic acid.

The chondroitin sulfate fractions contained both 4-sulfate and 6-sulfate. In the infrared spectra the 820/850 quotient decreased with increasing molecular weight of the chondroitin sulfate.

The present study reports the isolation and characterization of the glycosaminoglycans of costal cartilage from young, healthy guinea pigs. The purpose of this investigation was to obtain analytical data to serve as reference in current studies on the influence of vitamin C deficiency on the formation of the glycosaminoglycans in cartilage. As far as we are aware, no systematic investigation on the occurrence of different mucopolysaccharides in guinea pig rib cartilage has been reported in the literature. The isolation of mucopolysaccharide fractions, assumed to represent chondroitin sulfate, was reported by Reddi and Norström,[1] Bowness,[2] and Friberg.[3]

MATERIAL *

Rib cartilage was obtained from 30 young, male guinea pigs. The animals were supplied by a commercial breeder. They were kept in the laboratory under standardized

* *Abbreviations.* CPC=cetylpyridinium chloride, EDTA=ethylene diamine tetraacetic acid (disodium salt).

conditions for a period of 2—3 weeks. A semisynthetic diet [4] was given *ad lib.* The drinking water contained 0.1 % L-ascorbic acid.

The guinea pigs were sacrificed by exsanguination under ether anesthesia when they had reached a weight of 300—350 g. The cartilaginous portions of ribs II—VIII were dissected and removed, leaving a 2 mm wide zone at the costo-chondral junction and near the sterum. The perichondrium was removed by scraping and peeling. The cartilage was then cut into small pieces and pooled.

The pooled cartilage was placed in acetone overnight, and was then boiled in fresh acetone under reflux for 1 h. The cartilage was then dried in air. The total yield was about 3.5 g of dry, defatted cartilage.

ANALYTICAL METHODS

Hexosamines. The hexosamine content of isolated polysaccharide fractions was determined by the Elson and Morgan [5] reaction as modified by Blix,[6] and Antonopoulos *et al.*[7] Samples were hydrolysed with 6 N HCl for 8 h on a boiling water bath, with subsequent removal of the HCl *in vacuo* in a desiccator over sodium hydroxide pellets.

Separation of glucosamine and galactosamine was accomplished by chromatography on Dowex-50 ion exchange columns as described by Antonopoulos[8].

Hexuronic acids. Hexuronic acids were determined by the carbazol reaction according to Dische.[9]

Hexose. The anthrone method as modified by Goa [10] was used for the estimation of hexose.

Sulfate. Sulfate was determined by a modification of the benzidine method.[11]

Infrared analysis. Infrared spectrophotometry was made with a Unicam SP 200 instrument, using the KBr-pellet technique.

Susceptibility to testicular hyaluronidase. The method of Thunell [12] was used.

Analysis of the glycosaminoglycans on a microscale. The CPC-cellulose technique of Antonopoulos *et al.*[7] and the ECTEOLA-cellulose technique of Antonopoulos *et al.*[13] were followed.

Preparation and separation of the glycosaminoglycans on a macroscale. A 3 g sample of cartilage was digested with papain at 65°C for 3 h according to Scott [14] in 125 ml of a solution containing 0.05 M EDTA, 0.005 M cystein and 20 mg crystalline papain. The pH was approximately 7.

After digestion the solution was centrifuged at about 20 000 *g* for 30 min to remove insoluble material. The residue was washed twice with the digesting buffer, and the supernatants pooled.

To the pooled supernatants (about 200 ml) 100 ml of 1 % CPC were added. After standing for 36 h the precipitate was collected by centrifugation at about 3000 *g* for 15 min. The supernatant (A) was decanted and kept. The precipitate was suspended in a few ml of 1 % CPC solution. Propanol was added to a concentration of about 60 % (v/v), at which stage all of the precipitate was dissolved. The clear solution was applied to a 4 × 35 cm cellulose column prepared according to Antonopoulos *et al.*[15] After the polysaccharide solution had drained into the column, the walls of the column were washed with a few ml of propanol/CPC solution. The column was then eluted with the following solvents: 1 % CPC (1 l), 0.3 M NaCl in 0.05 % CPC (1 l), 0.6 M $MgCl_2$ in 0.05 % CPC (2 l), and, finally, 2 M $MgCl_2$ in 0.05 % CPC (1 l).

Fractions of 10—15 ml were collected with an automatic fraction collector. The tubes, containing the material eluted with 1 % CPC, were added to the supernatant, A. From this solution the polysaccharides were precipitated by the addition of 4 vol. of ethanol and 10 ml of a saturated solution of sodium acetate in water. After standing overnight the precipitate was collected by centrifugation at about 3000 *g* for 15 min. The precipitate was dissolved in a few ml of distilled water. A small insoluble residue was removed by centrifugation. The polysaccharides were again precipitated with ethanol in the presence of sodium acetate, centrifuged, and dried with ethanol and ether.

The presence of glycosaminoglycans in the fractions eluted with the salt solutions was detected by the addition of an equal volume of 1 % CPC to an aliquot of the fraction. Turbidity indicated the presence of glycosaminoglycans. The contents of the tubes

belonging to the same peak were pooled. Any turbidity in the pooled fractions was cleared by the addition of concentrated salt solution.

When NaCl was used as eluting solvent, the sodium salts of the glycosaminoglycans were precipitated with 3 vol. of 96 % ethanol, washed with absolute ethanol and ether, and weighed. In the case of the fractions eluted with $MgCl_2$ the glycosaminoglycans were similarly precipitated with ethanol. The magnesium salts of the glycosaminoglycans obtained were then converted to the corresponding sodium salts in the following way. The precipitate was dissolved in water, a small amount of NaCl was added, and the glycosaminoglycans were precipitated with 1 % CPC. After centrifugation (3000 *g*) the precipitate was suspended in a few ml of 1 % CPC solution and dissolved by the addition of propanol as described above. Sodium acetate was then added to the solution, and the sodium salt precipitated with 3 vol. of 96 % ethanol. The precipitate was collected by centrifugation, washed with absolute ethanol and ether, and weighed.

Fractionation of galactosaminoglycans. A 60 mg sample of the 0.6 M $MgCl_2$ fraction was further fractionated in the following way. The sample was dissolved in a few ml of 0.025 M Na_2SO_4 and precipitated with CPC. The precipitate was collected by centrifugation and dissolved in a few ml of propanol/CPC. This solution was applied to a cellulose-CPC column (2.5 × 30 cm), prepared according to Antonopoulos *et al.*[15] The column was then eluted with 500 ml of each of the following solutions: 1 % CPC, 0.35 M $MgCl_2$, 0.40 M $MgCl_2$, 0.45 M $MgCl_2$, 0.50 M $MgCl_2$ and, finally, 0.60 M $MgCl_2$. The salt solutions contained 0.05 % CPC.

The CPC fraction was discarded. To the $MgCl_2$ fractions 4 vol. of 0.05 % CPC were added. After standing for 24 h the precipitates were collected by centrifugation at 3000 *g* for 30 min. The precipitates were washed in 0.05 % CPC, dissolved in propanol/CPC and reprecipitated with 10 vol. of absolute ethanol in the presence of sodium acetate. After 18 h the sodium salts of the galactosaminoglycans were collected by centrifugation at 3000 *g* for 15 min. The precipitates were washed with absolute ethanol and ether, dried and weighed.

Gel filtration of galactosaminoglycans. Samples of 600—800 μg of the five galactosaminoglycan fractions were subjected to gel filtration as follows. A column of 1.1 × 175 cm was used. The column was packed with Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.2 M NaCl in 10 % (v/v) ethanol. This solution was also used for elution.

The samples were dissolved in 0.5 ml of the eluent and applied to the top of the column. Elution was then performed at a flow-rate of 4 ml/h. Fractions of 2 ml were collected.

The fractions were analyzed by the automated orcinol-sulfuric acid method of the Technicon Sugar Chromatography System (Technicon Chromatography Corp., Ardsley (Chauncey), New York).

Calculation of K_{av} values was made according to Laurent and Killander.[16]

RESULTS AND DISCUSSION

Total hexosamine content and the distribution of hexosamines between glycoproteins and glycosaminoglycans. The ECTEOLA-cellulose microcolumn technique[13] was used. The total amount of hexosamines was 5.3 % of the dry weight of the defatted costal cartilage. About 9 % thereof was derived from glycoproteins (aqueous and 0.02 M HCl fractions), whereas about 91 % was derived from glycosaminoglycans.

Identification of the glycosaminoglycans obtained on a macroscale. Table 1 shows the content of hexosamines, hexuronic acid, galactose and sulfate, and the digestibility with testicular hyaluronidase of the fractions.

The first fraction shows analytical data typical of keratan sulfate. Glucosamine was the predominant hexosamine. Galactose, but not hexuronic acid, was present. The infrared spectrum of this fraction showed characteristics of keratan sulfate.

Table 1. Analysis of the glycosaminoglycan fractions of guinea pig rib cartilage. Results expressed as percent of air dried material.

Fraction	Recovery mg	Glucos-amine	Galactos-amine	Uronic acid	Galac-tose	Sulfate	Ratio [a]	Sensitivity to hyal-uronidase
1 % CPC	25.4	17.2	4.0	0	25.6	15.9	1.40	
0.3 M NaCl	Traces	—	—	—	—	—	—	
0.6 M $MgCl_2$	584.5	0	24.6	29.4	0	15.6	1.18	+
2.0 M $MgCl_2$	1.4	23.0	Traces	0	24.5	23.4	1.90	—

[a] Molar ratio sulfate/hexosamine.

The 0.3 M NaCl fraction contained only traces of polysaccharide, which did not permit further analysis. Presumably, hyaluronic acid and/or low molecular chondroitin sulfates may have occurred in this fraction.

The 0.6 M $MgCl_2$ fraction was the major polysaccharide fraction. Galactosamine, uronic acid and sulfate occurred in approximately equimolar amounts. This fraction was almost completely digested with testicular hyaluronidase. Infrared analysis showed absorption bands at 720, 820, 850 and 928 cm^{-1}. The 820/850 quotient indicated that both chondroitin-4- and chondroitin-6-sulfate were present, possibly in the form of hybrides.

The final, 2.0 M $MgCl_2$, fraction contained hexosamine and galactose in a molar ratio close to one. Practically all of the hexosamine present was glucosamine. The molar ratio sulfate/hexosamine indicated a high degree of sulfation. This fraction was resistant to hyaluronidase digestion. The infrared spectrum indicated the presence of keratan sulfate.

Fractionation and characterization of the galactosaminoglycans. The analytical data obtained for the five galactosaminoglycan fractions are given in Table 2. Gel filtration patterns are shown in Fig. 1. The different fractions varied but little with regard to the molar ratios of uronic acid and sulfate to hexosamine. From the gel filtration analysis it may be concluded that the molecular weight of the chondroitinsulfates increased with increasing $MgCl_2$ con-

Table 2. Analysis of galactosaminoglycan fractions from guinea pig rib cartilage. Results expressed as percent of air dried material.

Fraction	Hexos-amine	Glucuronic acid	Sulfate	Molar ratio uronic acid/ hexosamine	Molar ratio sulfate/ hexosamine	IR 820/850	K_{av} G-200
0.35 M $MgCl_2$	16.0	21.0	11.0	1.22	1.35	1.10	0.58
0.40 M $MgCl_2$	24.8	33.0	13.1	1.23	1.00	0.99	0.49
0.45 M $MgCl_2$	27.0	35.0	15.0	1.20	1.04	0.91	0.39
0.50 M $MgCl_2$	24.2	35.0	15.3	1.33	1.17	0.90	0.34
0.60 M $MgCl_2$	24.2	32.0	15.2	1.22	1.17	0.86	0.19

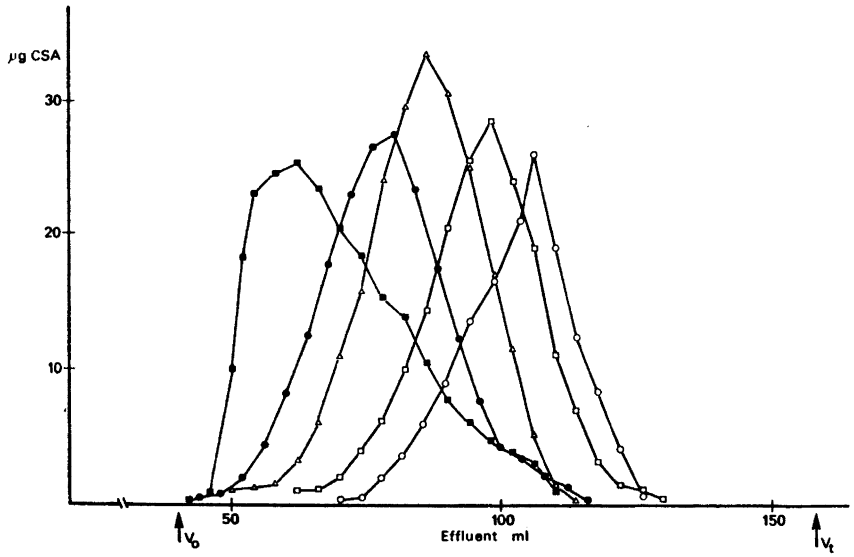

Fig. 1. Gel filtration on a column of Sephadex G—200 of galactosaminoglycan fractions eluted with salt solutions from a CPC-cellulose column. The CPC-cellulose column was eluted with 1 % CPC, 0.35 M $MgCl_2$ (○), 0.40 M $MgCl_2$ (□), 0.45 M $MgCl_2$ (△), 0.50 M $MgCl_2$ (●), and 0.60 M $MgCl_2$ (■). The salt solutions contained 0.05 % CPC.

centration. All but the 0.60 M $MgCl_2$ fraction gave symmetrical peaks at gel filtration. Infrared spectrophotometry showed a decreasing 820/850 quotient of the chondroitin sulfate eluted with higher concentrations of $MgCl_2$ (Table 2). This might indicate a higher proportion of chondroitin-4-sulfate with increasing molecular weight of the polysaccharide. Further investigations on this point are currently being performed and will be reported subsequently.

Quantitative fractionation of glycosaminoglycans on a microscale. Exact quantitative information on the composition of a mixture of glycosaminoglycans cannot be derived from fractionation on a macroscale. Hence, the papain digest of the rib cartilage was analyzed also by the microprocedure of Antonopoulos *et al.*[1]

The results are given in Fig. 2. This diagram shows that the bulk, about 80 %, of the hexamine-containing material, representing chondroitin-4- and chondroitin-6-sulfate (*cf.* Table 1), was eluted in the 0.25 M to 0.60 M $MgCl_2$ fractions. The amount of keratan sulfate may be calculated to be about 8 % by adding the 1 % CPC and 6 M HCl fractions, with subtraction of the value for glycoproteins. Hyaluronic acid and/or low molecular, low sulfated chondroitin sulfate constituted approximately 3 % of the total hexosamine content.

Concluding remarks. The major glycosaminoglycan fractions of guinea pig costal cartilage were found to be chondroitin-4- and chondroitin-6-sulfate and keratan sulfate. A somewhat similar polysaccharide pattern was reported for human rib cartilage by Meyer *et al.*[17] On the other hand, Hjertquist and

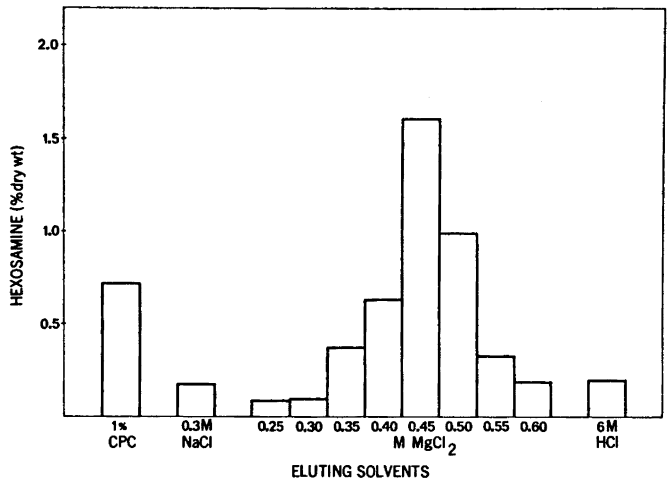

Fig. 2. Quantitative fractionation of the glycosaminoglycans of guinea pig rib cartilage on cellulose-CPC microcolumns.

Lemperg [18] did not find any chondroitin-6-sulfate or keratan sulfate in the costal cartilage of adult rabbits.

The metabolism of the different glycosaminoglycans in costal cartilage in normal and scorbutic guinea pigs, studied with labeled precursors, will be reported on in subsequent communications.

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