ISOLATION OF KERATOSULFATE FROM CHONDROMUCOPROTEIN OF BOVINE NASAL SEPTA

John D. Gregory* and Lennart Rodén

Institute of Biochemistry and Institute of Medical Chemistry, University of Uppsala, Sweden

Received July 18, 1961

Chondromucoprotein has usually been regarded as containing chondroitin sulfate as the only polysaccharide component. We have recently been studying its composition and structure with the aim of elucidating the nature of the protein-carbohydrate linkage. Enzymatic degradation has allowed the isolation of a small fragment containing amino acids, hexose, and chondroitin sulfate oligosaccharides. In addition, however, a fraction has been obtained with the properties of keratosulfate, with which this paper will mainly be concerned.

Chondromucoprotein was prepared from fresh cartilage (Malawista and Schubert, 1958), and proteinpolysaccharide-light (PP-L) was isolated by ultracentrifugation (Gerber, Franklin and Schubert, 1960). Analytical values for PP-L are given in Table I.

Hyaluronidase digestion. - PP-L (3.80 gm) was dissolved in 275 ml of water, 10 ml of 3 M sodium acetate buffer, pH 5.0, and 15 ml of 3 M NaCl. The pH was adjusted to 5 with 0.5 M NaOH (55 ml). The solution was stirred at 4° overnight, then incubated at 37-38° with stirring after the addition of 10 mg of hyaluronidase** (13, 600 Int. Units/mg). Turbidimetric assay (Dorfman, 1955) of the polysaccharide content showed that the digestion came to an end after about seven hours, with 15 % of the polysaccharide remaining undigested.

---

* National Science Foundation Senior Postdoctoral Fellow. Present address, The Rockefeller Institute, New York 21, N. Y.

** We are much indebted to Dr. Bertil Högberg, AB Leo, Hälsingborg, Sweden, for generous gifts of highly purified hyaluronidase.

The oligosaccharides were removed by gel filtration of 150 ml of digest on a column of Sephadex G-75 (ungraded*), 7 x 40 cm, equilibrated with 0.5 M sodium phosphate, pH 6.9. The hyaluronidase-degraded complex was recovered by dialysis against distilled water and freeze-drying. The combined yield from two gel filtrations was 726 mg. Assuming that PP-L is 15 % protein and that 15 % of the polysaccharide remained after digestion, the yield was 81 %. Analyses of the product are shown in Table I.

Papain digestion. - Four hundred mg of the hyaluronidase-degraded complex were dissolved in 10 ml of versenate-BAL buffer (Rosevear and Smith, 1961). To this was added 0.1 ml of a papain solution prepared by dialyzing 1 ml of crystalline papain suspension (Sigma Chemical Co., 17.5 mg/ml in 0.03 M cysteine) with 25 µl of BAL against several changes of the above buffer for 24 hours. The pH of the digestion mixture was adjusted to 6.35 with about 1 ml of 0.5 M NaOH, and incubation at 65° was begun. Similar additions of papain were made after 2, 8, and 23 hours, and the proteolysis was followed by means of the ninhydrin reaction on 5 or 10 µl aliquots (Moore and Stein, 1954). No further increase occurred after 20 hours, but the digestion was allowed to proceed for a total of 32 hours. A small precipitate (9.8 mg dry weight) was removed by centrifugation. The digest was concentrated to about 2 ml by freeze-drying and transferred with 2 ml of water to a column of Sephadex G-50, 2 x 103 cm, equilibrated with 0.05 M NaCl. The sample was followed by 0.05 M NaCl and 12-ml fractions collected. The carbazole reaction, the ultraviolet absorption, and the hexosamine content are shown in Figure 1.

Keratosulfate. - The peak emerging first had the properties of keratosulfate; the combined fractions of this peak (effluent volume 85-133 ml) were mixed with 4 volumes of ethanol and kept overnight at room temperature. The precipitate, washed with ethanol and ether and dried, weighed 58 mg.

---

* Pharmacia, Uppsala, Sweden (ungraded corresponds approximately to current medium grade).

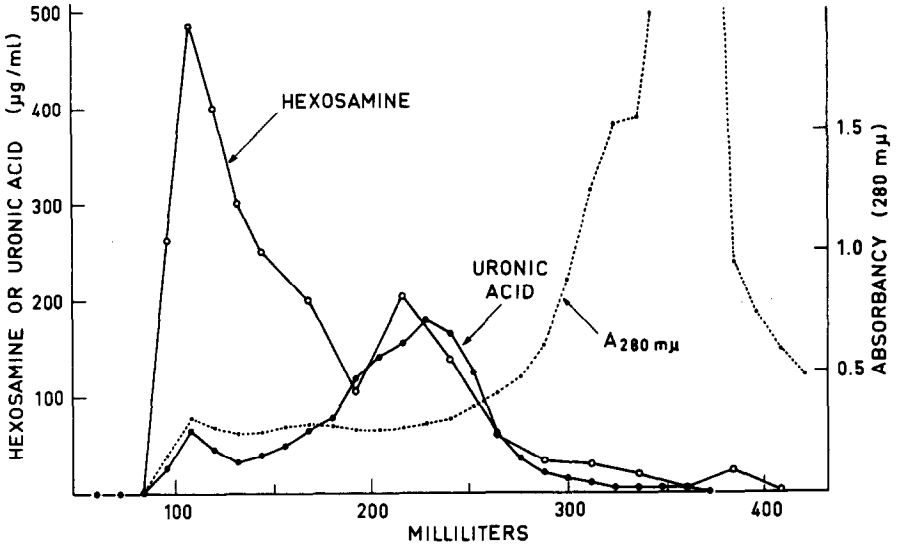

Fig. 1. Gel filtration of papain digest.

This product may have contained some papain (maximum of 7 mg if no denaturation had occurred), and the possibility was checked by passage of a sample in 0.2 M sodium phosphate buffer over Amberlite IRC-50 (XE-100) (Hill and Smith, 1960), to which test samples of papain were known to be adsorbed in the presence of corneal keratosulfate. A solution of 3.1 mg of keratosulfate from chondromucoprotein in 1.0 ml of water had an absorbancy of 0.488 at 280 mµ, and 21 % of this was retained by the resin. This corresponds to a maximum of 0.5 mg of papain in the whole keratosulfate sample.

Analytical values for the keratosulfate are shown in Table I; they may be compared with those quoted by Meyer et al. (1953) and Gardell (1957).

Additional confidence in the identity of this keratosulfate was derived from the behavior of its cetylpyridinium complex*, which had the

* The sample was kindly examined for us by Dr. Sven Gardell and Mr. Christos Antonopoulos, Serafimerlasarettet, Stockholm.

Table I

Analyses of various preparations

	PP-L	Hyaluronidase-degraded complex	Keratosulfate
Nitrogen[a]	4.8	10.1	4.8
Uronic acid[b]	22.6	5.3	< 2.7
Glucosamine-HCl[c]	2.5	9.8	26.6
Galactosamine-HCl[c]	21.8	4.8	3.1
Galactose[d]	7.0	12.3	33.5
Sulfate-sulfur[e]	4.7	2.0	4.8
$[\alpha]_D^{20}$	-	-	-1
Ash	24.3	7.9	-
Fucose[f]	< 0.7	-	< 1
Sialic acid[g]	2.2	4.2	7.7

Values are % on a dry-weight basis.

[a]Dumas method (Kirsten, 1957); [b](Dische, 1947); [c](Gardell, 1953); [d](Morris, 1948); galactose was qualitatively identified by paper chromatography in n-butanol-pyridine-water (3:2:1.5) after hydrolysis in 1 M sulfuric acid for 2 hours at 100°; [e]modification of Berglund and Sörbo (1960); [f](Dische and Shettles, 1948); [g]Bial method described by Odin (1959).

same solubility characteristics as those of keratosulfate from other sources.

Discussion. - Analysis of the keratosulfate preparation shows certain significant discrepancies. The presence of galactosamine suggests contamination with chondroitin sulfate or its fragments, and the high nitrogen value indicates residual peptide material, a situation that may allow investigation of the protein-keratosulfate linkage. Furthermore, the galactose content is higher than the glucosamine, and it may be involved in unsuspected ways in the total structure.

The presence of keratosulfate has been suggested by Partridge and Elsden (1961) in chondromucoprotein prepared in a somewhat different way. They have also presented some electrophoretic data indicating that keratosulfate and chondroitin sulfate are bound to the same protein molecules.

The existence of a high molecular-weight protein core has been offered as a reason for the fact that hyaluronidase does not digest the chondroitin sulfate of chondromucoprotein completely (Mathews and Lozaityte, 1958). The digestion of that component may actually be greater than commonly supposed, with keratosulfate (which is not attacked by hyaluronidase) largely making up the undigested acid polysaccharide.

References

Berglund, F., and Sörbo, B., Scand. J. Clin. Lab. Invest. 12, 147 (1960).
Dische, Z., J. Biol. Chem. 167, 189 (1947).
Dische, Z., and Shettles, L. B., J. Biol. Chem. 175, 595 (1948).
Dorfman, A., In "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 166. Academic Press, New York, 1955.
Gardell, S., Acta Chem. Scand. 7, 207 (1953).
Gardell, S., Acta Chem. Scand. 11, 668 (1957).
Gerber, B. R., Franklin, E. C., and Schubert, M., J. Biol. Chem. 235, 2870 (1960).
Hill, R. L., and Smith, E. L., J. Biol. Chem. 235, 2332 (1960).
Kirsten, W. J., Anal. Chem. 29, 1084 (1957).
Malawista, I., and Schubert, M., J. Biol. Chem. 230, 535 (1958).
Mathews, M. B., and Lozaityte, I., Arch. Biochem. Biophys. 74, 158 (1958).
Meyer, K., Linker, A., Davidson, E. A., and Weissman, B., J. Biol. Chem. 205, 611 (1953).
Moore, S., and Stein, W. H., J. Biol. Chem. 211, 907 (1954).
Morris, D. L., Science 107, 254 (1948).
Odin, L., Acta Soc. Med. Upsal. 64, 25 (1959).
Partridge, S. M., and Elsden, D. F., Biochem. J. 79, 26 (1961).
Rosevear, J. W., and Smith, E. L., J. Biol. Chem. 236, 425 (1961).