The Sulfated Mucopolysaccharides from Human Umbilical Cord

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SUMMARY

Umbilical cord was shown to contain chondroitin sulfate B and a small amount of glucosamine-containing sulfated mucopolysaccharide, in addition to hyaluronic acid and chondroitin sulfate C.

All of the hyaluronic acid and part of the chondroitin sulfate C could be extracted directly with dilute sodium chloride; the rest of the chondroitin sulfate C and the chondroitin sulfate B were released only after digestion with proteolytic enzymes.

The presence of hyaluronic acid in umbilical cord was established in Meyer's laboratory in 1936 (2), and its chemical and physical properties have been the subject of numerous reports. The details of these studies have been reviewed recently (3). In addition to hyaluronic acid, umbilical cord is known to contain sulfated mucopolysaccharides (2, 4, 5). In their systematic study on the mucopolysaccharides of various tissues, Meyer et al. (6), reported that umbilical cord contains chondroitin sulfate C or chondroitin 6-sulfate. On the basis of histochemical studies it has been proposed that umbilical cord also contains heparin (7).

In the course of other investigations in our laboratory it became apparent that umbilical cord contained a mixture of several sulfated mucopolysaccharides, some of which had not been recognized or characterized previously. The present study was undertaken to fractionate and purify the mucopolysaccharides, and to determine the nature, properties, and relative amounts of these substances.

EXPERIMENTAL PROCEDURE

Methods—Protein was assayed by the method of Lowry et al. (8). Hexosamines were determined by the Elson-Morgan reaction, as modified by Boas (9), after hydrolysis for 16 hours with 4 N HCl. Uronic acid was generally measured by the carbazole (10) and orcinol (11) procedures and in certain instances by the deoxyribonuclease (12). The anthone reaction (13) was used for the determination of hexose. Analyses for sulfate were made according to Muir (14) as modified by Cifonelli and Dorfman (15).

To determine digestibility by hyaluronidase, a mixture composed of 0.1 M acetate buffer (pH 6), 0.15 M NaCl, 1 mg of substrate, and 15 units of testicular hyaluronidase (Worthington) in a total volume of 1.6 ml was incubated at 37° for 2 hours. Aliquots were then assayed for terminal N-acetylmuramidine (16) and reducing sugar (17, 18).

Hexosamines in the mucopolysaccharides were identified on acid hydrolysates by the procedure of Stoffyn and Jeankow (19). The uronic acid moiety was identified as follows. A solution of 2 mg of sample in 0.4 ml of 2 N HCl was heated in a sealed tube at 100° for 1½ hours. The solution was cooled and rapidly evaporated to dryness at room temperature, and the residue was dissolved in 1 ml of water. The aqueous solution was passed through a column (8 x 20 mm) of Dowex 50-W in order to remove the hexosamine. After the eluate had been evaporated to a small volume, it was chromatographed on paper with solvent systems which clearly distinguished between glucuronic and iduronic acid (20, 21).

Infrared spectra were obtained with a Perkin-Elmer infrared spectrophotometer by the KBr pellet technique.

Extraction and Fractionation—A quantity (500 g) of human umbilical cord from which the blood clots were removed was homogenized with 1 liter 0.2% NaCl at 0-5° for 5 min. The viscous homogenate was diluted with an equal volume of 0.2% NaCl and shaken at 5° for 1 day. The solution was centrifuged and the supernatant fraction was collected. The extraction of the tissue residue was repeated five times, whereupon no additional polysaccharide was taken up. The solid remaining after these operations was designated as Fraction P (Fig. 1).

An aqueous solution of cetylpyridinium chloride (1%) was added to the extracts, and the resultant precipitates were collected and washed with 0.04 M NaCl. The precipitate containing the cetylpyridinium-mucopolysaccharide complex was extracted, successively, with six 200-ml portions of 0.4 M NaCl (Fraction SA), 1.2 M NaCl (Fraction SB), and 2.1 M NaCl (Fraction SC). After the extracts were concentrated to approximately half their original volume, the mucopolysaccharides were precipitated by the addition of 3 volumes of ethanol. The respective precipitates were collected by centrifugation, redissolved, and dialyzed against running water at 4° for 2 days. Lyophilization of the resulting solutions yielded 1.0, 0.5, and 0.04 g of Fractions SA, SB, and SC, respectively.
Purification and Isolation of Mucopolysaccharides—A solution of 500 mg of Fraction SA in 300 ml of water was passed through a column of Dowex 1-chloride. The column was washed with 1 liter of water and then eluted with 0.5 M NaCl. The effluent was collected on an automatic fraction collector in 20-ml fractions, until no carbohydrate-containing material was detectable. The collection of approximately 3 liters (Fraction SAA) was required. The column was then eluted successively with 1.2, 1.5, 2.0, and 3.0 M NaCl. Polysaccharides were found in the 1.2 M and 2.0 M eluates designated Eluate SAB and Eluate SAD, respectively. Each fraction was dialyzed exhaustively against water and concentrated to a small volume. Addition of 3 volumes of ethanol yielded precipitates which were collected by centrifugation, washed with ethanol, and dried.

When Fractions SB and SC were adsorbed on the anion exchange resin and eluted with NaCl solutions of increasing concentrations, there was no significant purification except for removal of part of the protein. An alternate procedure was therefore employed. A solution of 160 mg of Fraction SB was dissolved in 25 ml 0.04 M calcium acetate and incubated with 3 mg of Streptomyces griseus protease (Pronase, Calbiochem) at 37° for 2 days. The pH was maintained at 8. A small amount of toluene was added to prevent bacterial growth. At the end of the incubation, an equal volume of 10% sodium acetate was added and the mixture was shaken for 1 hour with Lloyd's reagent. The solid was removed by centrifugation and the supernatant was treated with chloroform-amyl alcohol according to the Sevag procedure (22) to remove residual protein. The clear aqueous layer was concentrated to 25 ml, and the polysaccharide was recovered by precipitation with 3 volumes of ethanol. The precipitate was dissolved in 25 ml of 0.2 M acetic acid containing 5% calcium acetate, and the mucopolysaccharides were isolated by fractional precipitation with ethanol. The major fractions were obtained with 35% ethanol (57 mg) and with 50% ethanol (53 mg), designated Fractions SB-35 and SB-50, respectively.

Fraction SC was purified in a similar manner. The major product was obtained with 50% ethanol (designated Fraction SC-50), 26 mg from 45 mg of Fraction SC.

Since further extraction of Fraction P with 0.2% NaCl yielded no additional mucopolysaccharide, the fraction was subjected to preliminary digestion with proteolytic enzymes. Fraction P was homogenized in 500 ml of water and digested first with pepsin at pH 2.5 for 2 days, and then with trypsin at pH 8.5 for 4 days. After the digestion period, 1 ml of 40% trichloroacetic acid was added per 3 volumes of the mixture, and the resultant precipitate was removed by centrifugation. The supernatant was treated with chloroform-amyl alcohol according to the Sevag procedure (22) to remove residual protein. The Sevag procedure (22) was collected on an automatic fraction collector in 20-ml fractions, until no carbohydrate-containing material was detectable. The collection of approximately 3 liters (Fraction SAA) was required. The column was then eluted successively with 1.2, 1.5, 2.0, and 3.0 M NaCl. Polysaccharides were found in the 1.2 M and 2.0 M eluates designated Eluate SAB and Eluate SAD, respectively. Each fraction was dialyzed exhaustively against water and concentrated to a small volume. Addition of 3 volumes of ethanol yielded precipitates which were collected by centrifugation, washed with ethanol, and dried.

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Fraction P-II was also treated with alkali to remove protein: 20 mg of Fraction P-II were dissolved in 2 ml of 0.5 N NaOH and kept under nitrogen atmosphere at 5° for 1 day. The solution was acidified to pH 5.5 with acetic acid and then treated in the same manner as described for the Pronase-treated material. This yielded a product (Fraction P-II*, 14.5 mg) which was precipitated from 35% ethanol.

RESULTS

The fractionation procedure employed in this study is outlined in Fig. 1. In the initial step the mucopolysaccharides were separated on the basis of their extractability from the tissue with 0.2% NaCl. The soluble fraction contained hyaluronic acid and sulfated mucopolysaccharides. Treatment of this mixture with cetylpyridinium chloride and extraction of the resultant precipitate with sodium chloride solutions of increasing molarity (23) yielded Fractions SA, SB, and SC. Fractionation of SA on Dowex 1 yielded Components SAA and SAB in addition to minute amounts of Component SAD. These products contained glucosamine as the only hexosamine moiety. The
analytical data are shown in Table I. The results on Fraction SAA conformed with the known data for hyaluronic acid. Its infrared absorption spectrum was identical with that of hyaluronic acid from synovial fluid and vitreous humor. This product was digested by testicular hyaluronidase at the same rate as hyaluronic acid from the other sources.

The sulfated Fractions SAB and SAD, like hyaluronic acid, were susceptible to digestion by testicular hyaluronidase. Fraction SAB contained equimolar amounts of glucosamine, glucuronic acid (carbazole reaction), and sulfur. Its infrared spectrum was similar to that of hyaluronic acid, except for an additional absorption maximum at 870 cm\(^{-1}\). Fraction SAD was similar to Fraction SAB, but was apparently contaminated with protein. These fractions may be either sulfated hyaluronic acid or a mixture of hyaluronic acid and heparin. The former alternative appears unlikely; however, the question remains to be resolved.

Fractions SB and SC both contained galactosamine as the only detectable hexosamine. These products contained a small but significant amount of protein, which was eliminated by digestion with Pronase. Subsequent fractionation of the calcium salts with ethanol according to the procedure of Meyer et al. (6) yielded Fractions SB-35, SB-50, and SC-50. Analyses of the products are given in Table II. The data for galactosamine, glucuronic acid, and sulfate conform with those for chondroitin sulfate A or C. The infrared absorption spectra of the three fractions were identical, all showing maxima at 1000 and 825 cm\(^{-1}\) characteristic for chondroitin sulfate C (24). After digestion with hyaluronidase, a positive reaction was obtained with the Morgan-Elson reagents, a reaction expected with chondroitin sulfate C but not with chondroitin sulfate A (25). These results, together with the specific rotation, lead to the conclusion that all three fractions are chondroitin sulfate C.

Proteolytic digestion of the tissue residue from the extraction with 0.2% NaCl (Fraction P), followed by ethanol fractionation of the calcium salts, yielded two major products, Fractions P-I and P-II. The hexosamine moiety in both of these was found to be galactosamine. Both products contained equimolar amounts of hexosamine, uronic acid (by the decarboxylation method), and sulfur. Fraction P-I showed an orcinol to carbazole ratio of 2:1 (Table III), characteristic for chondroitin sulfate B (26). Paper chromatography of hydrolysates showed that the uronic acid moiety was iduronic acid. A typical experiment with one of the solvents is shown in Table IV. About 50% of the protein was removed by digestion with Pronase (Fraction P-I', Table III). The material was not digested by hyaluronidase, and the specific rotation was highly negative. The conclusion that the mucopolysaccharide is chondroitin sulfate B was further substantiated by the identity of the infrared absorption spectrum with that of an authentic sample (characteristic maxima at 928, 860, and 840 cm\(^{-1}\)).

Fraction P-II was identified as chondroitin sulfate C on the basis of an authentic sample (characteristic maxima at 928, 860, and 840 cm\(^{-1}\)).
basis of analytical data (Table III), solubility of its calcium salt in aqueous ethanol, and infrared spectrum (maxima at 825 and 1000 cm\(^{-1}\)). This polysaccharide was digested with hyaluronidase, and the product gave a positive reaction for N-acetylhexosamine as well as for reducing sugar. Chondroitin sulfate A, which has a sulfate ester group on C-4, does not give a positive reaction after hyaluronidase digestion with the Morgan-Elson assay system (25).

Treatment of Fraction P-II with alkali resulted in the removal of almost all of the bound protein, and yielded product P-II'. The protein was also almost completely removed by digestion with Pronase. The product (P-II') was apparently identical with the chondroitin sulfate C obtained in the NaCl soluble fractions (see Table II). The difference in susceptibility to Pronase between the bound protein in chondroitin sulfate B (Table III) and that in chondroitin sulfate C probably reflects a difference in the individual amino acids or in their sequence. Neither mucopolysaccharide fraction, P-I or P-II, showed any ultraviolet absorption at 280 nm, indicating little or no aromatic amino acids in the peptide portion of these fractions.

The amounts of the different mucopolysaccharide fractions which were isolated from 500 g of umbilical cord are shown in Table V.

**DISCUSSION**

The results of the present study show that the principal sulfo mucopolysaccharides of umbilical cord are chondroitin sulfate C and chondroitin sulfate B. There is also present a small amount of a glucosamine-containing sulfo mucopolysaccharide which has not been characterized. Most of the chondroitin sulfate C could be extracted directly from the tissue with dilute sodium chloride, while the rest of this material, and all of the chondroitin sulfate B, require preliminary digestion with pepsin and trypsin. This difference in extractability might suggest a compartmentalization of the mucopolysaccharides between the structural components of umbilical cord, or a variation in the proteins to which they are bound.

Umbilical cord is composed primarily of mesenchyme, termed Wharton's jelly. A very small, yet significant, component is vascular material, i.e. two arteries and a vein. The isolation procedure described in previous reports (2, 4–6) and those employed in the present work do not allow one to specify the original location of the mucopolysaccharides in umbilical cord. It is generally assumed that they are derived from Wharton's jelly, and this assumption is supported by results from histochemical studies (7). Although the possibility that a fraction of the mucopolysaccharides isolated might be from vascular material is not to be excluded, it seems unlikely in view of the amounts and proportions of mucopolysaccharides known to be present in human aorta (26) and other blood vessels (27). Furthermore, if vascular components made any significant contribution to the mucopolysaccharides isolated, there should have been an appreciable amount of heparin sulfate among the products obtained after proteolytic digestion. The fact that the latter was not isolated or detected strongly suggests that the source of the mucopolysaccharides is Wharton's jelly. However, further study is required to resolve this question, since the content of mucopolysaccharides in blood vessels has been described only for adult and not for embryonic tissue.

**Acknowledgments**—We wish to express our gratitude to Dr. Martin Stone and the members of the Department of Obstetrics and Gynecology for making available to us the umbilical cords used in these studies. We would also like to thank Dr. Sara Schiller for samples of authentic mucopolysaccharides, and for her help in the sulfate determinations.

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