Carbohydrate Research, 28 (1973) 103–108

Note

Reaction between carbohydrates and sulfuric acid. Part IV. Polymerization and sulfation of D-glucose by sulfuric acid

KINZO NAGASAWA AND YUKO INOUE

School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5 chome, Minato-ku, Tokyo 108 (Japan)

(Received June 8th, 1972; accepted in revised form, October 25th, 1972)

Dissolution of D-glucose in conc. sulfuric acid at a temperature below 0° forms a mixture of sulfated products of D-glucose[1]. Takiura *et al.*[2] have reported that the treatment of D-glucose, D-galactose, D-mannose, or D-fructose in cold conc. sulfuric acid resulted in the formation of the trisulfate of the corresponding monosaccharide as a main product. The present paper reports the characterization of a polymer of sulfated D-glucose residues formed by the treatment of D-glucose with cold conc. sulfuric acid.

RESULTS AND DISCUSSION

D-Glucose was treated with conc. sulfuric acid and the resulting reaction mixture of sodium salts was fractionated by dialysis (Table I). After two successive dialyses, a nondialyzable product was recovered (Fr. III) in 16.0% yield. As shown in Table I, the sulfur contents of Fr. I–IV were similar, and each fraction migrated at the

TABLE I

PHYSICAL AND CHEMICAL CONSTANTS OF THE REACTION PRODUCTS OF D-GLUCOSE WITH CONC. SULFURIC ACID AND OF REFERENCE POLYSACCHARIDE SULFATES

Polymer	*Sulfur* (%)	*Sulfate ratio*[a]	$\overline{M}_w$	$[\eta]$	$[\alpha]_D$ *in water*[b] (°)	*Yield*	
						(*g*)	(%)[c]
Fr. I	19.50	2.60				4.5[d]	36.4
Fr. II	20.06	2.81			49.7 (22.2)	6.4[d]	49.4
Fr. III	19.16	2.48	1600[e]	0.0092	59.3 (23.8)	0.6[f]	16.0
Fr. IV	19.42	2.59		0.0035	49.0 (25.0)	1.3[f]	33.7
Depolymerized dextran sulfate	18.94	2.41	7000–8000[g]	0.0126	120.9 (24.0)		
Cellulose sulfate	19.53	2.61			−15.6 (22.0)		

[a]Ratio of sulfate group to D-glucose residue. [b]Temperature (in degrees) given in parentheses. [c]Calculated from the amount of sodium salt obtained from 5 g of D-glucose and the degree of sulfate substitution. [d]Weight obtained from 5 g of D-glucose. [e]Determined by the light-scattering method. [f]Weight obtained from 2 g of Fr. II. [g]Determined by viscometry at the Research Laboratory of Seikagaku Kogyo Co. Ltd.

same rate as a single spot on paper electrophoresis, which suggests that the sulfation was relatively uniform.

The reducing value of Fr. III was 0.87% of that of D-maltose indicating a number-average molecular weight ($\overline{M}_n$) of 41,600. A comparison of the specific viscosity of Fr. III with that of depolymerized dextran sulfate suggests that the true molecular weight of Fr. III is considerably smaller than 41,600. For an accurate estimation of the degree of polymerization, the weight-average molecular weight ($\overline{M}_w$) of Fr. III was determined by the light-scattering method and it was found to be 1600 ($\overline{D.P.}$ *ca.* 4). In the fractional precipitation of Fr. III with ethanol (Fig. 1), the least soluble fraction corresponds to 10% of Fr. III (Fr. III-a, Fig. 1), and it was chromatographed on Sephadex G-25 with Fr. III and IV. The elution patterns show a relatively homogeneous distribution of molecular size of each sample, and their position of elution agrees with their rate of dialysis or of precipitation as shown in the Experimental section. The rate of migration of Fr. I, III, and IV on paper chromatograms corresponds to the rate of dialysis, and the nature of the spots indicates heterogeneity of the molecular size.

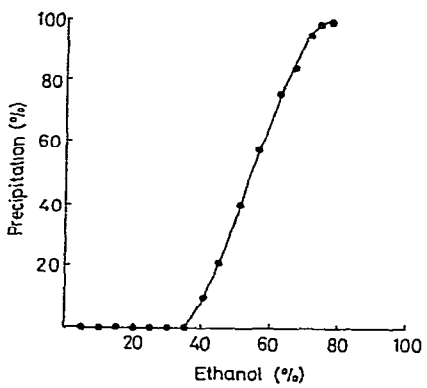

Fig. 1. Fractional precipitation by ethanol of polymerized, sulfated D-glucose (Fr. III). A solution of Fr. III (100 mg) in 0.15M sodium chloride (10 ml) was precipitated by step-wise addition of ethanol. Fr. III-a (9.95 mg) was precipitated at an ethanolic concentration of 40%.

The properties of Fr. III are in agreement with the transformation of D-glucose by conc. sulfuric acid into a mixture of polymers of sulfated D-glucose residues. Since the reaction conditions used in the present work are very similar to those reported by Takiura *et al.*[2], the D-glucose 1,3,6-trisulfate described by those authors may be one of the components of Fr. I, which was found by paper chromatography to be highly heterogeneous.

For the determination of the linkage of the sulfate groups and of the sulfated D-glucose residues, Fr. III was hydrolyzed with 0.25M hydrochloric acid at 95°. The liberation of inorganic sulfate from Fr III was compared to that of depolymerized dextran sulfate and cellulose sulfate (see Table II). The result suggests that the linkage of the sulfate groups of Fr. III is partially different from that of the two last

named materials. The rapid release of sulfate groups from Fr. III at the beginning of the hydrolysis would indicate a possible substitution of the reducing end with a sulfate group, which would explain the low reducing power of the fraction. The titration data are in agreement with the presence of monoesters. The reducing values of Fr. III, depolymerized dextran sulfate and cellulose sulfate after hydrolysis (see Table III) suggest that Fr. III is possibly formed by a condensation-polymerization involving the hydroxyl groups of the D-glucose residues. Examination of the p.m.r. spectra of Fr. III and of related D-glucan sulfates showed for Fr. III a broad peak at 5.4–5.5 p.p.m. which is attributed to the anomeric proton; maltose sulfate, lactose sulfate, and amylopectin sulfate gave signals attributed to the anomeric proton, at 5.56, 5.08, and 5.63 p.p.m., respectively. A crude estimate of the ratio of the peak area of the anomeric proton to the sum of the area of the other protons (at 3.9–4.4 p.p.m.) suggested for Fr. III the presence of one glucosidic linkage per D-glucose unit.

Since no other component besides D-glucose and inorganic sulfate was detected on the chromatogram of the hydrolysate of Fr. III, no structural change occurred in the D-glucose residues during the reaction with conc. sulfuric acid. It is known that the acid-catalyzed polymerization of unprotected D-glucose molecules gives strongly dextrorotatory products having a highly branched structure[3]. The moderately positive, specific rotation of Fr. III, suggests that the glycosidic linkages consist of a random mixture of α- and β-D configurations. The degree of both polymerization and the sulfation of the D-glucose molecules may be expected to depend on the concentration of the sulfuric acid. In fact, lowering this concentration resulted in both lower yield and lower degree of sulfation of the nondialyzable fraction: in 90% sulfuric acid, 2.02 g and S 16.97%; in 85% sulfuric acid, 0.60 g and S 12.28%; and in 80% sulfuric acid, 0.02 g and S 7.06%, respectively, starting from 5.0 g of D-glucose.

EXPERIMENTAL

Material. — Conc. sulfuric acid was of reagent grade and contained 96% of sulfuric acid by weight. Organic solvents and reagents, which were all special reagent grade, were used without further purification. Depolymerized dextran sulfate (sodium salt: S 18.94%, $\overline{M}_w$ 7,000–8,000 by viscosity analysis) was obtained from Seikagaku Kogyo Co. Ltd., Tokyo. Sodium cellulose sulfate (S 19.53%, $\overline{M}_n$ 162,500) was prepared from microcrystalline cellulose "Avicel" and sodium amylopectin sulfate (S 16.91%, $\overline{M}_n$ 64,500) from amylopectin by the conc. sulfuric acid method[4]. Sodium maltose sulfate (S 18.78%) and sodium lactose sulfate (S 19.67%) were prepared by the sulfation of maltose and lactose, respectively, with triethylamine–sulfur trioxide in *N,N*-dimethylformamide for 24 h at 0°. Potassium D-glucose 6-sulfate was obtained from Sigma Chemical Co., U. S. A.. Type C-65 and 36/32 of Visking tube were used for dialysis.

Analytical procedure. — All the samples to be analyzed were dried *in vacuo* in the presence of phosphorus pentaoxide for 3 h at 80°. The sulfur content was determined by the Dodgson turbidimetric method[5]. The number-average molecular

weight ($\bar{M}_n$) was calculated from the value obtained by the hypoiodite method[6]. The weight-average molecular weight ($\bar{M}_w$) was determined by the light-scattering method with a Shimadzu light-scattering photometer, as described in a previous paper[4]. A modified Ubbelhode viscometer[7] was used for the determination of intrinsic viscosity at 25±0.1° in 0.9% (w/v) sodium chloride[8]. P.m.r. spectra were measured at 35° with a Varian T-60 NMR spectrometer operating at 60 MHz on a 10–15% solution of the sample in deuterium oxide containing sodium 4,4-dimethyl-4-silapentanesulfonate as an internal standard. Chemical shifts are expressed in p.p.m. on the δ-scale.

Chromatographies. — Descending paper chromatography was performed on Toyo Roshi No. 51 paper in butyl alcohol–acetic acid–water (50:12:25, v/v) and t.l.c. on cellulose powder "Avicel-SF" plates. Paper electrophoresis was performed on Toyo Roshi No. 51 paper with a buffer solution (pH 5.8) consisting of pyridine (5 ml), acetic acid (1 ml), butyl alcohol (5 ml), and water (250 ml), at a potential of 23 V/cm for 25 min. The spots were detected with aniline hydrogen phthalate and with ammoniacal silver nitrate. The spots of sulfated materials were also detected by staining with 1% (w/v) ethanolic Toluidine Blue.

Treatment of D-*glucose with conc. sulfuric acid.* — Anhydrous D-glucose (5 g) was added gradually to conc. sulfuric acid (15 ml) at 0° to form a homogeneous solution which was kept for 2 h at the same temperature under stirring. The reaction mixture was poured into ice–water, neutralized with cold 15% sodium hydroxide, and dialyzed against deionized water (1 liter) for 18 h, and then against running tap water for 30 h in a type C-65 Visking tube.

After being tested for the absence of inorganic sulfate, the nondialyzable fraction was concentrated to 10–20 ml at a temperature below 40° and centrifuged. The supernatant was added to ethanol (10 vol.), and the precipitate formed was filtered off, washed successively with ethanol and ether, and dried in the presence of phosphorus pentaoxide for 2 h at 80° (Fr. II, 6.4 g).

The dialyzable fraction obtained from the dialysis against deionized water (1 liter) was concentrated to *ca.* 100 ml. A 10% solution of barium acetate was added to remove inorganic sulfate and, after centrifugation, the supernatant solution was applied to a column (2.5 × 16 cm) of Dowex 50 (W-X2, 50–100 mesh, Na^+) to remove barium ions. The effluent and washings were combined, evaporated at a bath temperature below 40° to 10 ml, and then centrifuged. The dialyzable product was precipitated and dried as described for the nondialyzable product (Fr. I, 4.5 g).

Fr. II (2 g) was further dialyzed three times against deionized water (2 l) for 15 h each time. Both the nondialyzable fraction and the combined dialyzates were purified as just described to give the second nondialyzable product (Fr. III, 0.6 g) and the second dialyzable product (Fr. IV, 1.3 g).

Treatment of D-*glucose with dilute sulfuric acid.* — Samples of D-glucose (5 g) were treated with sulfuric acid of various concentrations (90, 85, and 80%, w/w) for 2 h at 0°, and each reaction mixture was treated as just described. The first nondialyzable fractions (which correspond to Fr. II) were analyzed for yield and sulfur content.

Fractional precipitation of polymerized sulfated D-*glucose.* — A solution of the sample (Fr. III, 100 mg) in 0.15M sodium chloride (10 ml) was precipitated at 0° with ethanol in constant increments of 5% ethanol concentration. After each addition, the turbid solution was kept for 20 h at 0°, and the precipitate was centrifuged off, washed with ethanol, and dried in air and then *in vacuo* in the presence of phosphorus pentaoxide for 16 h at room temperature. Fraction III-a was precipitated at 40% ethanol concentration (Fig. 1).

Sephadex-gel filtration. — A solution of the sample (Fr. III-a, Fr. III, and Fr. IV, 2.0 mg each; or D-glucose 6-sulfate, 1.7 mg) in 0.1M sodium chloride (1 ml) was applied to a column (1.5 × 89 cm) of Sephadex G-25 (fine) and eluted with 0.1M sodium chloride at a flow rate of 10 ml/h. Gel-filtration of Blue Dextran was separately performed. Fractions of 2.2 ml each were collected, and the concentration of each fraction was determined by the Toluidine Blue-binding technique[9], except that of Blue Dextran and D-glucose 6-sulfate which was determined by the absorbancy at 660 nm and by the 3,6-dinitrophthalic acid method[10], respectively. The elution volumes (ml) observed for each sample were 63.0 for Blue Dextran, 66.0 for Fr. III-a, 67.2 for Fr. III, 72.4 for Fr. IV, and 110.0 for D-glucose 6-sulfate.

Acid hydrolysis of polymerized sulfated D-*glucose, depolymerized dextran sulfate, and cellulose sulfate.* — A solution of the samples (Fr. III, depolymerized dextran sulfate, and cellulose sulfate, 2.0 mg each) in 0.25M hydrochloric acid (10 ml) was heated at 95°. Aliquots (1 ml) of the solution were neutralized with 0.25M sodium hydroxide (1 ml) and analyzed by the Dodgson turbidimetric method[5] for sulfate and the Somogyi method[11] for reducing value.

TABLE II

INORGANIC SULFATE LIBERATED (%) ON ACID HYDROLYSIS

	Time (min)		
	15	*30*	*180*
Fr. III	41.5	67.9	96.0
Depolymerized dextran sulfate	12.3	71.0	96.3
Cellulose sulfate	28.3	64.7	97.0

TABLE III

REDUCING VALUES (%) FOLLOWING ACID HYDROLYSIS

	Time (h)		
	1	*5*	*24*
Fr. III	36.7	86.7	97.2
Depolymerized dextran sulfate	18.8	62.0	99.5
Cellulose sulfate	33.5	86.4	94.5

Titration of polymerized sulfated D-*glucose.* — A solution of the sample [Fr. III, 15.60 mg (93.17 μmoles of ester sulfate) and 15.07 mg (90.00 μmoles of ester sulfate)] was passed through a column (0.5 × 6.5 cm) of Dowex 50 (W-X8, 20–50 mesh, H^+) to remove sodium ions. The effluent and washings were combined and titrated with 0.01M sodium hydroxide to give 91.74 and 89.03 μequiv., respectively.

Identification of hydrolysis products from polymerized sulfated D-*glucose.* — A solution of the sample (Fr. III, *ca.* 20 mg) in 0.25M hydrochloric acid (5 ml) was heated for 15 h at 100°. An aliquot of the hydrolyzate was neutralized with 0.25M sodium hydroxide and analyzed by paper electrophoresis, the spot of inorganic sulfate being detected with the rhodizonate reagent. The remaining hydrolyzate was treated with an excess of Amberlite IR-4B (OH^-) to remove both chloride and inorganic sulfate ions and evaporated *in vacuo*. The residual solution was analyzed by t.l.c. in 5:5:1:3 (v/v) pyridine–ethyl acetate–acetic acid–water (R_F 0.38) and in 6:4:3 (v/v) butyl alcohol–pyridine–water (R_F 0.27); observed for D-glucose: R_F 0.39 and 0.27, respectively.

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. T. Kamata for the determination of molecular weight by the light-scattering method and thank Mr. S. Shinkai and Miss H. Yoshidome for technical assistance.

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