BRANCHED α-1,4-GLUCAN FROM THE GASTROPOD MOLLUSK Strombus gigas

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From the mantle of the Caribbean Sea gastropod mollusk Strombus gigas a bioglycan containing carbohydrates (73%), protein (22%), and lipid (about 5%) components has been isolated. It has been shown by hydrolysis, periodate oxidation, and methylation that the bioglycan is a D-glucan constructed of α-1,4-bound D-glucose residues and has side chains of D-glucose residues linked by α-1,6-bonds.

It is known that marine invertebrates are sources of polysaccharides and glycoconjugates (bioglycans) which possess immunostimulating activity [1]. The physiological activity of a bioglycan isolated from the mantle of the gastropod mollusk Strombus gigas, which is found widely in the seas of the Caribbean basin, has been reported previously [2]. In the present paper we give information on the characterization and study of the chemical structure of its carbohydrate component.

The homogeneity of the bioglycan was shown by ultracentrifugation and gel filtration on Sepharose CL-2B. D-Glucose was identified as the sole monosaccharide in the polysaccharide by the paper and gas-liquid chromatography of a hydrolysate. The high specific rotation of the polysaccharide, $[\alpha]_D^{20}$ +135° (c 0.35; water) showed the α-configuration of the glycosidic bonds. The bioglycan contained 22% of protein, its total carbohydrate content being 73% and its lipid content about 5%.

The weight-average molecular weight of the bioglycan (~2 MDa) was determined by gel filtration in Sepharose CL-2B using standard dextrans (T-250, T-500, and T-2000).

The glucan was methylated by Hakomori's method with completing methylation by Purdie's method. The completeness of methylation was checked with the aid of IR spectroscopy.

In a hydrolysate of the permethylated glucan the following glucose derivatives were identified in the form of acetates of methyl glycosides and of methylated derivatives of polyol acetates by GLC and chromato-mass spectrometry: 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-O-methylglucoses in molar ratios of 1.6:5.8:1 (acetates of the polyols of methylated derivatives). The results of the chromato-mass spectrometry of derivatives of acetates of polyols from the methylated glucan are given below:

Methylated monosaccharide (polyol acetate)	Molar ratios	Type of bond
2,3,4,6-Me_4 Glc	1,6	Glc·*p*(1→
2,3,6-Me_3 Glc	5,8	→4)—Glc·*p*—(1→
2,3-Me_2 Glc	1	→4,6)—Glc·*p*—(1→

The methylation results showed the branched nature of the carbohydrate chain of the glucan. The presence of 2,3,6-tri-O-methylglucose showed that in the main chain of the glucan the D-glucopyranose residues were linked by 1,4-glucosidic bonds. The presence of 2,3-di-O-methylglucose served as proof of the fact that some of the D-glucose residues of the main chain had branching at C-6. The results of methylation were also confirmed by those of ^{13}C NMR spectroscopy.

In the ^{13}C NMR spectrum of the glucan there were two signals in the region of anomeric C-atoms at δ 100.6 and 99.2 ppm. The ring C-atoms participating in the formation of the glycosidic bonds resonated at 78.4 and 68.4 ppm. These results show the presence of α-1,4- and α-1,6-glucosidic bonds in the glucan.

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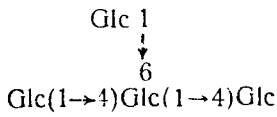
The glucan was oxidized with 0.05 M sodium metaperiodate at 4°C for 10 days. The oxidized polysaccharide was reduced with sodium tetrahydroborate and the polyalcohol formed was hydrolyzed with sulfuric acid. The hydrolysate was analyzed by paper and gas-liquid chromatography in the form of polyol acetates; glycerol, erythritol, and glucose were identified in molar ratios of 2.6:10.6:1. The glycerol and erythritol were formed from terminal and 1,4-bound D-glucose residues, respectively. The detection of glucose in the hydrolysate of the polyalcohol did not agree with the results of the methylation of the initial glucan and can be explained by incomplete oxidation as a consequence of features of the macrostructure of the glucan or by the presence of a small number of 1,3-glucosidic bonds.

Weak acid hydrolysis of the polyalcohol led to the formation of a complex mixture which was separated on a column of TSK-Gel HW-40 followed by paper chromatography.

As a result, tetrasaccharide (1) with R_{Glc} = 0.45; $[\alpha]_D$ +23° (c 0.2; water) was isolated. In a hydrolysate of the permethylated tetrasaccharide, 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-O-methylglucoses were identified in a ratio of 2:1:1, which corresponds to the following branched structure for it:



Treatment of the glucan with saliva amylase led to the formation of glucose and a number of oligosaccharides, which were separated with the aid of paper chromatography. As a result, two oligosaccharides were isolated in the individual state: disaccharide (2) with R_{Glc} = 0.63; $[\alpha]_D$ +102.9° (c 0.6; water) and trisaccharide (3) with R_{Glc} = 0.46; $[\alpha]_D$ +127° (c 0.5; water).

In hydrolysates of the permethylated oligosaccharides (2) and (3), 2,3,4,6-tetra- and 2,3,6-tri-O-methylglucoses were identified in ratios of 1:1 and 1:2, respectively. The methylation results show that oligosaccharide (2) was maltose, while (3) was maltotriose. The high specific rotations of the oligosaccharides indicated the α-configurations of the glycosidic bonds.

The ultrasonic treatment of the glucan led to the formation of glucose and a series of oligosaccharides, which were separated by paper chromatography. As a result, disaccharide (4) (R_{Glc} = 0.64), trisaccharide (5) (R_{Glc} = 0.42), and pentasaccharide (6) (R_{Glc} = 0.22) were isolated.

Oligosaccharides (4) and (5) were shown to be identical with oligosaccharides (2) and (3), respectively. In a hydrolysate of permethylated oligosaccharide (6) 2,3,4,6-tetra- and 2,3,6-tri-O-methylglucoses were identified in a ratio of 1:4 and, consequently, it was a maltopentaose.

The degraded glucans remaining after the action of amylase (GAs) and after ultrasonic treatment (GUs) were studied by the methylation method. In hydrolysates of the permethylated GAs and GUs 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3- and 2,6-di-O-methylglucoses were identified.

The 2,6-di-O-methylglucose was detected in trace amounts, and its formation may show a possible presence of a small number of 1,3-bound branches in the glucan molecule or be a consequence of an incomplete methylation of the polysaccharide.

Thus, the biopolymer from Strombus gigas consists of a high-molecular-weight D-glucan having a main carbohydrate chain of α-1,4-bound D-glucose residues and side chains attached to approximately each seventh D-glucose residue of the main chain by α-1,6-glucosidic bonds. The presence of a small number of side chains attached by α-1,3-bonds to the main carbohydrate chain is not excluded.

EXPERIMENTAL

Materials and Methods. For paper chromatography we used Filtrak FN-3,12 or 15 paper and the solvent system 1) pyridine–butan-1-ol–water (4:6:3). Monosaccharides were detected with aniline phthalate or a solution of silver nitrate.

Gel chromatography was performed on columns of Sephadex G-75 and G-25, TSK-Gel HW-40, and Sepharoses CL 2B and 6B, using distilled water as eluent. Gas-liquid chromatography was performed on a Pye-Unicam 104 chromatograph (United Kingdom) with a flame-ionization detector on a colum (0.4 × 150 cm) filled with Gas Chrom Q (100-120 mesh) impregnated with 3% of QF-1. Optical rotations were measured on a Perkin-Elmer 141 polarimeter.

IR spectra were taken on a UR-20 spectrophotometer. Chromato-mass spectrometry was performed on a LKB-900 instrument (Sweden), using the same column as in GLC. ^{13}C NMR spectra were obtained on a Bruker WM-250 instrument. A sample of the glucan was investigated in the form of a solution in D_2O at 80°C. Methanol was used as internal standard. Chemical shifts are given relative to tetramethylsilane.

Ultracentrifugation was performed on a MOM 3170 analytical ultracentrifuge with schlieren optics at 6000 rpm in water at 20°C.

The ultrasonic treatment was carried out with a UZDN-2T ultrasonic dispenser at 0°C in a current of argon at 22 kHz.

Preparation of the Bioglycan. The gastropod mollusk Strombus gigans was collected in the basin of the Caribbean Sea. The mantle of the freshly trapped mollusk was extracted with aqueous salt solution. After dialysis and lyophilization, the biopolymer was obtained in the form of a white powder (yield 5.7 g on 100 g weight of the crude initial material), and was stored at +4°C.

Molecular-Weight Determination. A solution of the glucan (8 mg) in water (1 ml) was deposited on a column (1.6 × 36 cm) of Sepharose CL-2B and was eluted with water at the rate of 7.5 ml/h. Fractions with a volume of 2.5 ml were collected, and aliquots of each fraction were analyzed by the phenol/sulfuric acid method [3] and by Lowry's method [4]. The molecular weight of the glucan was determined from a calibration curve plotted with the use of the standard dextrans D-250, T-500, and T-2000.

Monosaccharide Composition. The glucan or a degraded derivative of it (3 mg) was hydrolyzed with 1 N sulfuric acid in a sealed tube at 100°C for 5 h. The hydrolysate was neutralized with barium carbonate and was treated with a cation-exchanger in the $[H^+]$ form. The monosaccharide composition was investigated with the aid of paper chromatography in system 1 and by the GLC of polyol acetate and aldononitrile acetate derivatives.

Methylation. The glucan (40 mg) or a degraded glucan (GA – 12 mg; GU – 6 mg) was methylated by Hakomori's method [5] and then by Purdie's method [6]. The completeness of methylation was checked with the aid of IR spectroscopy.

The completely methylated compound was heated with 0.5 N hydrogen chloride in absolute methanol at 100°C for 7 h. The acid was eliminated by repeated evaporation with methanol in vacuum at 37°C. The methyl glycosides obtained were acetylated and were investigated by GLC and chromato-mass spectrometry [7].

A fully methylated compound (1 mg) was heated with 90% HCOOH (1 ml) in a sealed tube at 100°C for 2 h. The formic acid was eliminated by evaporation and the residue was hydrolyzed by 0.25 M H_2SO_4 (1 ml) at 100°C for 10 h. After neutralization of the acid, polyol acetates were obtained [8]. The partially methylated polyol acetates were analyzed by GLC and by chromato-mass spectrometry.

Periodate Oxidation; Smith Degradation. The glucan (107 mg) was oxidized with 0.05 M $NaIO_4$ (21.5 ml) in the dark at 4°C for 10 days. The excess of periodate was decomposed with ethylene glycol (5 ml), and the solution was dialyzed against distilled water. The polyaldehyde was reduced with $NaBH_4$ (10 mg) for 24 h. The excess of tetrahydroborate was decomposed with acetic acid, and the solution was dialyzed and lyophilized; yield 88 mg (on the weight of the glucan taken). The polyalcohol obtained (3 mg) was hydrolyzed with 1 N sulfuric acid at 100°C for 4 h. The hydrolysate was analyzed by paper chromatography (PC) and GLC in the form of the corresponding polyol acetates. Glycerol, erythritol, and glucose were identified in molar ratios of 2.7:10.6:1.

The polyalcohol (61 mg) was hydrolyzed with 0.25 N sulfuric acid (12 ml) at 20°C for 24 h and, after the neutralization of the acid ($BaCO_3$) the products were chromatographed on a column (1.8 × 54 cm) of TSK-Gel HW-40 at a rate of elution of 12 ml/h. Aliquots of each fraction were analyzed by the phenol/sulfuric acid method and by paper chromatography. The fractions containing oligosaccharides were treated with β-amylase and the products were

purified with the aid of preparative PC. As a result, an oligosaccharide with R_{Glc} 0.45 (5 mg) was isolated.

Enzymatic Hydrolysis. A solution of 550 mg of the glucan in 55 ml of water was treated with 1.2 ml of saliva amylase and the mixture was incubated at 37°C for 3 h. The enzyme was inactivated by boiling (5 min) in the water bath, and the solution was concentrated and precipitated with ethanol. The supernatant was separated off and, after evaporation, two oligosaccharides were detected by TC with R_{Glc} 0.65 and 0.46. The oligosaccharides were separated with the aid of preparative PC. The yields of the oligosaccharides were 17.9 and 18.3 mg, respectively. The degraded glucan was isolated after chromatography on a 1.5 × 75 cm column of Sephadex G-75.

Treatment with Ultrasound. The glucan (1 g) in distilled water (100 ml) was treated with ultrasound at 22 kHz for 10 min. The solution was dialyzed, and the dialysate was concentrated (165 mg). Glucose and a series of oligosaccharide fragments were identified in the dialysate with the aid of PC. On subsequent treatment of the glucan with ultrasound the yield of dialysate amounted to 70 mg.

The mixture of oligosaccharides was separated on Whatman 3MM paper, as a result of which oligosaccharides were obtained with R_{Glc} 0.22, 0.42, and 0.64. The yields of the oligosaccharides were 16, 23, and 5 mg, respectively. The oligosaccharides were methylated and investigated as described above.

The degraded glucan (GU) was purified by gel filtration on a column of Sephadex G-75 (1.5 × 75 cm).

CONCLUSIONS

A bioglycan (mol. wt. 2 MDa) has been isolated from the mantle of the Caribbean Sea gastropod mollusk Strombus gigas, which contains carbohydrate (73%), protein (22%), and lipid (about 5%) components. On acid hydrolysis of the bioglycan, D-glucose was identified as the sole monosaccharide. From the results of methylation, periodate oxidation, Smith degradation, and enzymatic and ultrasonic treatment, it follows that the main carbohydrate chain consisted of α-1,4-bound D-glucose residues and side chains of D-glucose residues attached by α-1,6-bonds. The α-configuration of the glycosidic bonds follows from the high positive specific rotation of the bioglycan.

The ^{13}C NMR spectrum confirmed the structure of the polysaccharide chain determined chemically.

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