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METHYLATION STUDIES OF THE POLYSACCHARIDES RESULTING FROM SEQUENTIAL SMITH-DEGRADATIONS OF THE GALACTAN FROM THE SNAIL *Strophocheilus oblongus**

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ABSTRACT

When the galactan from the albumen glands of the snail *Strophocheilus oblongus* was submitted to three Smith-degradations, the degraded polysaccharide, isolated in 6% yield, was much more linear. Methylation analysis showed that the Smith-degraded polysaccharide gave an increased percentage of 2,4,6-tri-, decreased percentages of 2,3,4,6-tetra- and 2,4-di-, and a large variation in the amount of 2,3,4-tri-*O*-methyl-D-galactose. The sugars in the polysaccharide which result in the formation of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-D-galactose are destroyed in subsequent degradation procedures. The above observations suggest that the degradation by periodate oxidation proceeds *via* non-reducing end-groups and through some internal residues that are exposed as the degradation proceeds. As a result of the overall process, new non-reducing end-groups are formed and new (1 → 6)-linked D-galactose residues are then exposed. The isolation of glycosides of low molecular weight supports the suggestion that the molecule, in the main, is sequentially degraded from the external layers.

INTRODUCTION

In an earlier publication, the structures assigned to galactans isolated from various species of mollusc were discussed[1–4]. Some are composed mainly, if not entirely, of β-(1 → 3)- and β-(1 → 6)-linked D-galactopyranose residues[4–5]. Others also contain (1 → 2)-glycosidic linkages[6] and L-galactopyranosyl groups[1,4,7,8], and all so far investigated have highly ramified structures. O'Colla[3] applied the Barry-degradation procedure to the galactan from the albumen glands of *Helix pomatia*, and showed that the alternative structure proposed by Baldwin and Bell[1] for this polymer was an over-simplification and that the polymer has a dichotomous structure.

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A highly branched structure for the galactan elaborated by the albumen glands of *Biomphalaria glabrata* has been proposed[4]. More recently, Duarte and Jones[5] isolated a thrice-degraded polysaccharide (8% yield) using the Smith-degradation procedure, and concluded that the original galactan was multibranched and showed features that differentiated it from the galactans of others molluscs[1–7].

We now report on the structure of the galactan from the albumen glands of *S. oblongus*, one of the largest snails that is not a vector in transmission of *Bilharzia*.

RESULTS AND DISCUSSION

The polysaccharide extracted from the albumen glands of *S. oblongus* was purified as previously described[5] using cetyltrimethylammonium bromide in the presence of borate buffer (pH 8.5).

The low, positive values of $[\alpha]_D^{25}$ for the degraded polysaccharides obtained after the first (+21°) and the second degradation procedures (+10°) support the suggestion[5] that the original galactan is built up mainly, if not entirely, of β-linked D-galactopyranosyl residues.

Chromatography of the original polysaccharide and the degraded polymers on Sephadex G-200 indicated that the products were not separable into discrete fractions and that the molecular-weight distribution broadened as the number of oxidation steps increased.

When the original and degraded polysaccharides were intensively methylated (15 times) and then hydrolysed, the previously reported[5] trace of 2,3,6-tri-*O*-methyl-D-galactose was not detected. After the first Smith-degradation, methylation analysis of the degraded polysaccharide after methanolysis showed that the amount of 2,4-di-*O*-methyl-D-galactose fell from 43 to 26%, paralleling the percentage changes of non-reducing end-groups (Table II). The amount of 2,4,6-tri- and 2,3,4-tri-*O*-methyl-D-galactose increased from 11 to 30% and from 3 to 18%, respectively. These changes are interpreted as a loss of end-groups or of some internal residues with the concurrent increase in D-galactose residues that are linked through positions 1,3 and 1,6 only. The latter type of sugar, which has free-hydroxyl groups on C-2, C-3, and C-4, is susceptible to further periodate oxidation with the formation of formic acid (Table I). Since gross fragmentation of the polysaccharide did not occur, it was concluded that most of the sugar units lost were removed by a "peeling-type reaction" from the periphery of a large molecule.

Methylation analysis of the twice-degraded galactan showed a further drop in end-groups (26 to 18%), a large increase in (1 → 3)-linked residues (30 to 52%), and a drop in the formation of (1 → 6)-linked D-galactose residues. A decrease in disubstituted D-galactose residues was also observed (26 to 18%) (Table II). These results are best explained by assuming that the molecules are formed of a main chain of (1 → 3)-linked sugars containing disubstituted sugars (linked through positions 1, 3, and 6). The ramified chains are of variable length and are linked to those branching points (disubstituted residues) producing either (1 → 3)- or (1 → 6)-linked sugars.

TABLE I

SMITH-DEGRADATION PRODUCTS OF THE GALACTAN

Degradation number	*Periodate-oxidation number*	*Non-dialyzable sample (g)*	*Dialyzable sample (g)*	*Mol of periodate per residue of galactose*	*Mol of formic acid per residue of galactose*	*Ratio of periodate to formic acid*
	First	3.90 (100%)	—	0.94	0.44	2.1:1
First	Second	2.00 (51%)	1.23	0.80	0.37	2.1:1
Second	Third	1.07 (27%)	0.90	0.60	0.27	2.1:1
Third	Fourth	0.24 (6%)	—	—	—	2.1:1

TABLE II

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED GALACTAN AND DEGRADED POLYMERS

O-*Methylated methyl* D-*glycosides*	T *values*[a]	*Mole %*[b]		
		A[c]	B[c]	C[c]
2,3,4,6-Tetra-	2.05	43	26	18
2,4,6-Tri-	5.15m 6.06s	11	30	52
2,3,4-Tri-	9.8	3	18	12
2,4-Di-	25.5m 29.9s	43	26	18

[a]Relative to that of 2,3,4,6-tetra-*O*-methyl-β-D-glucoside; [b]Determined by g.l.c. analysis (column *c*). [c]Polysaccharide: *A*, original; *B*, once degraded; *C*, twice degraded. Key: s, strong; m, moderate.

TABLE III

SMITH-DEGRADATION PRODUCTS OF THE ORIGINAL AND DEGRADED POLYMERS

Alditol acetates	T *values*[a]	*Mole %*[b]		
		A[c]	B[c]	C[c]
Glycerol	0.09	45	34	33
Galactitol	3.12	55	66	67
Galactitol:Glycerol	—	1:2	2:1	2:1

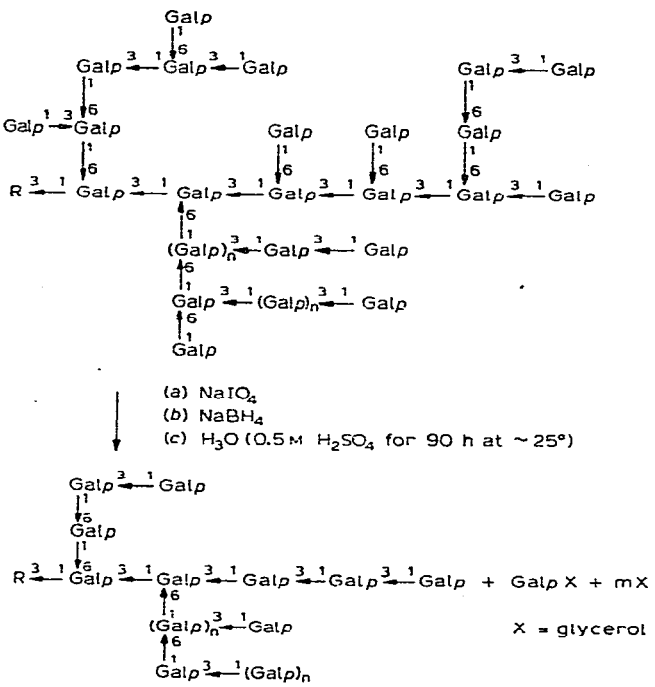
[a]Relative to that of L-arabinitol penta-acetate. [b]Determined by g.l.c. analysis (column *d*). [c]Polysaccharide: *A*, original; *B*, once degraded; *C*, twice degraded.

TABLE IV

ANALYSIS OF DIALYZABLE SAMPLES FROM SEQUENTIAL SMITH-DEGRADATIONS OF THE GALACTAN

Alditol acetate	T *values*[a]	*Mole %*[b]		
		A[c]	B[c]	C[c]
Glycerol	0.09	94.00	71.00	67.00
Galactitol	3.12	6.00	29.00	33.00
Galactitol:Glycerol	—	0.06	0.40	0.50

[a]Relative to that of L-arabinitol penta-acetate. [b]Determined by g.l.c. (column *d*). [c]Polysaccharide: *A*, original; *B*, once degraded; *C*, twice degraded.



Scheme 1.

The decrease of periodate uptake and formic acid liberated from the degraded polysaccharide (Table I) agrees well with the increase in the proportion of (1→3)-linked residues observed as the degradation proceeds (Table II). In accordance with this fact, the degraded polymers, when analyzed by the Smith-degradation procedure (Table III), contained a high proportion of D-galactose residues resistant to periodate oxidation. During the sequential Smith-degradations (Table IV), dialyzable fractions were obtained which contained large proportions of glycerol and derivatives of D-galactose and glycerol. These products were purified by chromatography on

Sephadex G-25 and by preparative p.c. The D-galactose derivatives were glycosides, as they were unnaffected by sodium borohydride. The ratio of D-galactose to glycerol was ~5:1 and 4:1 in two of major fractions isolated. The low ratio of galactitol to glycerol (Table IV) observed in the analysis of dialyzable fractions from the sequential Smith-degradations of the galactan also supports the suggestion that the original polymer is degraded mainly from the non-reducing end-groups and from some internal residues on the periphery of the molecule. The structure of these fractions will be described elsewhere.

To summarize, the mechanism of sequential Smith-degradations of the galactan is such that the non-reducing end-groups that are formed in one step are destroyed in the subsequent step of the process. This causes a growing increase of (1 → 3)-linked residues with the resultant formation of a more-linear polymer. This process is illustrated in Scheme 1 (β-D-galactopyranose residues only), which also shows the cleavage and appearance of new 1,6-linkages during one step of the oxidative degradation.

EXPERIMENTAL

General. — Free-boundary electrophoresis was performed with a Perkin–Elmer 38 A apparatus and 0.05M sodium borate buffer (pH 9.2) at 20 mamp and 150 V. Electrophoresis on cellulose acetate strips was performed with the apparatus of Fanem Ltd., according to the method of Dudman and Bishop[9]. I.r. spectra were determined for KBr discs (1%) with a Beckman IR-8 spectrophotometer. Optical rotations were measured with a Perkin–Elmer model 141 polarimeter at 25°.

G.l.c. (F & M chromatograph model 810R-12, flame ionization) of sugar derivatives was performed with helium as carrier gas (55 ml/min). The following columns were used: (*a*) 15% of butane-1,4-diol succinate polyester on 80–100 mesh acid-washed Celite (column 120 × 0.4 cm i.d.) at 185°; (*b*) 10% of polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] on 80–100 mesh acid-washed Celite (column 120 × 0.4 cm i.d.) at 180°; (*c*) 14% LAC-4R-886 on 80–100 mesh (D.M.S.C.) Chromosorb W (column 100 × 0.4 cm i.d.) at 160°; and (*d*) 1:1 (v/v) of a mixture of 10% butane-1,4-diol succinate polyester on 60–80 mesh Chromosorb W and 10% (w/w) of Apiezon M on silver-coated Chromosorb W (60–80 mesh) (column 120 × 0.4 cm i.d.) at 175°. Column (*c*) was used for quantitative analysis of the methylated sugars by the triangulation procedure[10]; *T* values are related to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside, and they were also compared with pure samples of methylated sugars[5]. Column (*d*) was used to determine the sugars as their alditol acetates[11], and the *T* values are related to that of D-arabinitol penta-acetate[12].

P.c. was performed by the descending method on Whatman No. 1 or 3MM papers, with (*e*) 1:5:3:3 (upper layer) benzene–butan-1-ol–pyridine–water, (*f*) 8:2:1 ethyl acetate–pyridine–water, (*g*) 9:2:2 ethyl acetate–pyridine–water, and detection with alkaline silver nitrate[13]. Mobility is given as R_{GAL} (free sugars). T.l.c. was performed on silica gel (Merck) with (*h*) 9:1 benzene–methanol, (*i*) 20:1 benzene–ethyl acetate, and detection with 5% ethanolic sulphuric acid at 150°.

Acetylation of the methylated hexitols was performed with acetic anhydride–pyridine (1:1.3) for 4 h at 100° or acetic anhydride–70% perchloric acid (140:1) for 25 h at 25°.

Hydrolysis of the galactan was carried out in sealed ampoules with 0.5M sulphuric acid for 5 h at 100°, followed by neutralisation with barium carbonate. The total sugar was determined by the phenol–sulphuric acid procedure[14]. M.p.s. were determined with the apparatus HMK 69/268 L. Reducing substances were detected with the phenyltetrazolium reagent[15]. Glycerol was identified by the standard procedure as its tris(*p*-nitrobenzoate)[16] (m.p. 193–195°); Nunn and von Holdt[17] reported m.p. 191–193°.

Isolation and purification of the polysaccharide. — The procedure followed that given by Duarte and Jones[5] (36% yield, $[\alpha]_D^{25}$ +25° (*c* 0.96, water)). The product of hydrolysis of the original galactan and of the degraded polysaccharides contained only D-galactose (crystalline from ethanol; $[\alpha]_D^{25}$ +80.5°; galactitol hexa-acetate, m.p. 170–172°).

Periodate oxidation of the galactan. — The original and degraded polysaccharides were oxidized with 0.01M sodium metaperiodate (50 ml/100 mg of sample). Portions of the solution were withdrawn at intervals and analyzed for periodate uptake[18] and yield of formic acid[19,20] (Table I). After periodate oxidation, samples of polysaccharide (original and degraded polymers) were submitted to the Smith-degradation procedure[21,22]. The products obtained from this process were analyzed by g.l.c. as alditol acetates[11] (Table III).

Sequential Smith-degradations of the galactan. — The polysaccharide (3.9 g) was oxidized with 0.05N sodium metaperiodate (500 ml) in the dark at 0–2° during 120 h. Excess of periodate was reduced with ethylene glycol (10 ml) during 2 h, the solution was dialyzed against distilled water (5 × 2 l) and then concentrated under reduced pressure, and the concentrate was reoxidized during 30 h under the conditions described above (but using only 250 ml of periodate solution). [The substances that passed through the dialysis sacs were non-carbohydrate (no reaction with the phenol–sulphuric acid and phenyltetrazolium reagents); the residue in the dialysis sac contained the polysaccharide.] The resulting polyaldehyde (3.5 g/100 ml) was reduced with sodium borohydride (4.8 g). After 48 h, excess of borohydride was decomposed by addition of 2M acetic acid. The solution was dialyzed and concentrated, and the polymer was again reduced with sodium borohydride (to avoid cyclic acetal formation) for 48 h as described above. After decomposition of excess of sodium borohydride, the solution gave a negative test with the phenyltetrazolium reagent, indicating that the reduction was complete. During the reduction, boric acid was added at intervals to keep the pH at ~10, in order to minimize the chance of β-elimination reactions. The resulting polyalcohol (3.4 g) was hydrolyzed with 0.5M sulphuric acid (500 ml) at 25° during 60 h. After dialysis against distilled water (10 × 2 l), the solution of the non-dialyzable portion was concentrated *in vacuo* and the resulting syrup was hydrolyzed again with 0.5M sulphuric acid (250 ml) at 25° for 30 h. A sample of the non-dialyzable portion (5 mg) was hydrolysed with 0.5M sulphuric

acid at 100° during 5 h. The solution was neutralized ($BaCO_3$), and the products were isolated in the usual way, acetylated, and then analyzed by g.l.c., (alditol acetates). Since no glycerol was detected (only D-galactose) in this hydrolyzate, the cleavage of the full acetals (alcoholic derivatives) of the polysaccharide was therefore considered complete after 90 h of hydrolysis (Table I) with dilute mineral acid at room temperature.

To examine the possibility that the polysaccharide had undergone unexpected fragmentation during the Smith-type degradations, its elution pattern on a column of Sephadex G-200 (120 × 2.5 cm) was examined. The polysaccharide was eluted with water, and the effluent was examined for sugar content by the phenol–sulphuric acid procedure[14]. No evidence of gross fragmentation could be observed. However, there was a broadening of the elution curve, indicating a change in the distribution of molecular size, and possibly the shape, of the molecule.

The periodate-oxidation technique may lead to erroneous results when applied to some polysaccharides, because of incomplete oxidation of the polysaccharide due to the intermediate formation of intramolecular hemiacetal links[23–25]. In order to check the oxidation results of the galactan, it was submitted twice to the sequence periodate oxidation and reduction with sodium borohydride, with intermediate dialysis. No significant increase in periodate oxidation resulted (0.02 mol of periodate/mol of "anhydro sugar"), and it was therefore concluded that structures resembling those described by Painter[23,24] were not formed.

The uptake of periodate and formation of formic acid were also determined for the degraded polysaccharide (first degradation, see Table I). This polymer was also methylated by different procedures (see methylation procedures, and Table II). The products obtained from periodate oxidation, borohydride reduction, and hydrolysis (0.5M sulphuric acid for 5 h at 100°) were analyzed by g.l.c. as alditol acetates (see Table III). Analysis of the degraded polymer on the Sephadex column (G-200) showed that no major fragmentation to smaller polysaccharide molecules had occurred. The twice-degraded polymer was submitted to a third Smith-type degradation and isolated as described above. The products of these sequential degradations showed no major fragmentation into smaller polysaccharide molecules. The results obtained from non-dialyzable samples are shown in Tables I, II, and III,

The dialyzable fragments. — The solution containing the dialyzable fragments from the first oxidation was neutralized ($BaCO_3$), centrifuged, and concentrated. A portion of the residue (50 mg) was reduced with sodium borohydride (3 × 50 mg), and the product was hydrolyzed (50 mg, 5 ml of 0.5M sulphuric acid at 100° during 5 h). The sugars were reduced ($NaBH_4$) and the mixture of alditol acetates was analyzed by g.l.c. The dialyzates from the second and third oxidations were examined similarly, and the results are recorded in Table IV. Portions of the dialyzate were also examined by p.c. [solvent (*e*)] after reduction by sodium borohydride. Various non-reducing oligosaccharides of glycerol (mono- and poly-hexosyl-glycerol) were detected, as well as ethylene glycol (from glycolaldehyde) and glycerol. D-Galactose and reducing oligosaccharides were not detected. The dialyzates from the first

and second oxidations were fractionated on a column of Sephadex G-25 (120 × 2.5 cm) with water, and by preparative p.c. [solvent (*f*)], to give two subfractions (I and II). Each fraction was hydrolyzed separately, and the products were reduced, acetylated, and then analyzed by g.l.c. The ratio of galactitol–glycerol was 4:1 for fraction I, and 5:1 for fraction II. Portions (15 mg) of these fractions were also treated with sodium borohydride, hydrolyzed, and acetylated, and the products examined by g.l.c. Galactose and glycerol acetates were detected; no galactitol hexa-acetate was present.

Methylation analysis of the galactan and degraded polymers. — The polysaccharide (200 mg) was methylated four times by the Haworth[26] procedure, and twice more in tetrahydrofuran solution with methyl sulphate and powdered sodium hydroxide[27,28] as described by Duarte and Jones[5]. Methylation was completed by dissolving the partially methylated products in methyl sulphoxide (10 ml) containing sodium hydroxide (2.0 g), followed by addition of methyl sulphate (10 ml) with vigorous stirring at ambient temperature during 8 h. The solutions were then neutralized (3M sulphuric acid), dialyzed against distilled water, and concentrated. Each resulting syrup was methylated three times more, using the same process. Finally, the products were thrice methylated by the method of Purdie and Irvine[29] and isolated in the usual manner. I.r. spectroscopy showed the absence of OH groups in the molecule. The yield was 60% after 15 methylations. Before the first methylation process, the polysaccharide was treated with sodium borohydride to reduce the reducing-end groups of the molecule in order to minimize the chance of β-elimination reactions. The methylation reactions were also carried out under nitrogen.

The methylated polymer (0.12 g) in 7% methanolic hydrogen chloride (2 ml) was heated for 6 h at 100° in a sealed tube. The cooled solution was neutralized with silver carbonate and concentrated, and the syrupy mixture of methyl glycosides was analyzed by g.l.c. using columns (*a*) and (*c*) (Table II); the *T* values of these methyl glycosides agree well with those reported by other authors[10,30] and with authentic samples[5].

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