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SOME STRUCTURAL STUDIES ON THE FUCOGALACTAN FROM EGG MASSES OF THE SNAIL *Ampullarius* sp.*

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ABSTRACT

The heteropolysaccharide isolated from egg masses of the snail *Ampullarius* sp. is composed of D-galactose and L-fucose residues. The fully methylated polysaccharide gave 2,3,4,6-tetra-(27 mole%), 2,4,6-tri-(5 mole%), 2,3,4-tri-(26 mole%), 3,4,6-tri-(11 mole%), and 2,4-di-*O*-methyl-D-galactose (29 mole%), and 2,3,4-tri-*O*-methyl-L-fucose (2 mole%). The results of periodate oxidation and Smith degradation are in satisfactory agreement with the methylation data, and indicate that the fucogalactan is a highly branched polysaccharide containing D-galactose and L-fucose as non-reducing end-groups and branching points at C-3 and C-6 of the D-galactose residues. In addition to a preponderance of (1→6) over (1→3) linkages, the fucogalactan contains part of the internal residues of D-galactose linked (1→2), which differentiates it from the polysaccharides of other molluscs that have been studied previously. No appreciable difference between polysaccharides isolated from freshly collected egg masses and from albumen glands of the *Ampullarius* sp. was observed.

INTRODUCTION

Galactose-containing polysaccharides have been isolated from albumen glands and egg masses of several different species of molluscs. Although this type of polysaccharide was isolated[1] as early as 1885, structural studies were made only recently.

The polysaccharides isolated from either albumen glands ($[\alpha]_D$ −14°)[2] or egg masses ($[\alpha]_D$ −23°)[3] of *Helix pomatia* contained both D- and L-galactose residues[4–6]. O'Colla, applying the Barry-degradation procedure[7] to the galactan from albumen glands of *Helix pomatia*, showed that the alternative structure proposed by Baldwin and Bell[4] for this polymer was an over-simplification and that the polymer had a dichotomous structure. A highly branched structure for the galactan elaborated by the albumen gland of *Biomphalaria glabrata* has been proposed[8]. The product of the acid fragmentation of the galactan from *Helix pomatia* egg masses has been studied[9,10],

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and the galactan of *Strophocheilus oblongus* is a highly branched polysaccharide[11] and shows features that differentiate it from the galactans of *Helix pomatia*[4] and *Biomphalaria glabrata*[8].

Although no other kinds of polysaccharide have been found in the freshly collected egg masses, it is known that glycogen is biosynthesised near the middle of the embrionary life of the snails[12,13], when the content of galactan decreases to 22%.

The present paper reports some structural features of the heteropolysaccharide isolated from the egg masses and albumen gland of *Ampullarius* sp.

RESULTS AND DISCUSSION

The content of polysaccharide in egg masses of *Ampullarius* sp. (6.4% dry weight) is less than that found in the egg masses of other molluscs[12–14]. The water-soluble polysaccharide extracted from an acetone-dried powder, after removal of the protein under mild conditions (proteolytic enzyme and Sevag procedure), was purified as its cetyltrimethylammonium complex in the presence of a borate buffer (pH 8.5). Examination of the recovered polysaccharide by electrophoresis and different molecular-sieve chromatographic procedures indicated that it was homogeneous and suitable for structural investigations. The polymer contained no nitrogen (protein) or carbonyl groups (i.r.). The product of acid hydrolysis of the polymer was shown by g.l.c. and other analyses to be composed of D-galactose {98 μmole%, $[\alpha]_D^{25}$ +80.5° (*c* 1, water)} and L-fucose (2 μmole%). No evidence was found for the presence of L-galactose or galactofuranosyl residues. The analysis corresponded to a heteropolysaccharide and it can therefore be classed as a fucogalactan. The optical rotation, $[\alpha]_D^{25}$ +51° (*c* 0.76, water), differs greatly from the value (−21°) found for the galactan from *Helix pomatia*. The present data were not enough to conclude that the fucogalactan is built up entirely of β-linked D-galactose and L-fucose residues.

The periodate consumption (1.10 mole/mole of anhydro sugar) of, and the formic acid produced (0.47 mole/mole of anhydro sugar) from, the polysaccharide indicated that 47% of the D-galactose residues had been oxidized and, after reduction with borohydride, produced glycerol [non-reducing end-groups or (1→6)-linked D-galactose residues]; 16% of the D-galactose residues consumed periodate but did not produce formic acid [(1→2)- or (1→4)-linked D-galactose residues)], and 37% of the D-galactose residues were resistant to periodate oxidation, indicating substitution at C-3 (see below). The results of Smith degradation [glycerol (72 μmole%), D-galactose (28 μmole%), absence of erythritol] excluded the presence of (1→4)-linked D-galactose residues. On the other hand, the high yield of glycerol was consistent with the presence of (1→2)-linked D-galactose residues.

Methanolysis of the methylated fucogalactan and g.l.c. of the resulting glycosides (Table I) revealed 2,3,4,6-tetra- (27 mole%), 2,4,6-tri- (5 mole%), 2,3,4-tri- (26 mole%), and 2,4-di-*O*-methyl-D-galactose (29 mole%), 2,3,4-tri-*O*-methyl-L-fucose (2 mole%), and one component (*X*) (11 mole%) with retention time (*T*) of 5.62. On the basis of periodate-oxidation and Smith-degradation data, and g.l.c. of

TABLE I

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED POLYSACCHARIDES (EGG MASSES AND ALBUMEN GLANDS)

Product	T *values*[a]			*Mole percentage*	
	Column (a)	*Column* (b)	*Column* (c)	*Egg masses*	*Albumen glands*
2,3,4,6-Tetra-*O*-methyl-D-galactoside	1.80	1.58	2.00	27.00	24.50
2,4,6-Tri-*O*-methyl-D-galactoside	—	—	5.16 m 6.08 s	5.00	6.00
3,4,6-Tri-*O*-methyl-D-galactoside	4.10	2.22	5.62	11.00	16.00
2,3,4-Tri-*O*-methyl-D-galactoside	6.60	2.90	9.90	26.00	28.00
2,4-Di-*O*-methyl-D-galactoside	15.00 m 16.90 s	3.80 m 4.40 s	26.00 m 30.00 s	29.00	25.00
2,3,4-Tri-*O*-methyl-L-fucoside	0.76	0.73	0.76	2.00	0.50

[a]Relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside. Key: s, strong; m, moderate.

its alditol acetate, *X* was indicated to be a 3,4,6-tri-*O*-methyl-D-galactose derivative. In the mixture of methylated reducing sugars obtained on acid hydrolysis of the methylated fucogalactan, only that derived from *X* was oxidised by periodate and gave a 2,3,5-tri-*O*-methyl-D-lyxose derivative (g.l.c. of the methylated alditol acetate and methyl glycoside, Table II). The mass spectrum of the alditol acetate derived from *X* showed primary fragments at *m/e* 45, 161, 189, 205, and 233, which proved[15] the 3,4,6-positions of the methoxyl groups.

TABLE II

G.L.C. OF METHYLATED SUGARS DERIVATIVES OBTAINED FROM THE METHYLATED FUCOGALACTAN (EGG MASSES)

Derivative	T *values*[a,b]			
	A	B	C	D
2,3,4,6-Tetra-*O*-methyl-D-galactoside	2.00	2.00	—	—
3,4,6-Tri-*O*-methyl-D-galactoside	5.62	—	5.62	—
2,3,4-Tri-*O*-methyl-D-galactoside	9.90	9.90	—	—
2,4,6-Tri-*O*-methyl-D-galactoside	5.16m 6.08s	5.16m 6.08s	—	—
2,4-Di-*O*-methyl-D-galactoside	26.00m 30.00s	26.00m 30.00s	—	—
2,3,4-Tri-*O*-methyl-L-fucoside	0.76	0.76	—	—
2,3,5-Tri-*O*-methyl-D-lyxoside	—	0.68	—	0.68

[a]Relative to that of methyl 2,3,4,6-tetra-*O*-methyl-*β*-D-glucoside. Key: s, strong; m, moderate. [b]*A*, Methanolysis products of the methylated fucogalactan; *B*, the acidic-hydrolysis products of the methylated fucogalactan were oxidized ($NaIO_4$) and submitted to the Fischer glycosidation procedure; *C*, compound *X* isolated (t.l.c.) from a mixture of methyl methylated sugars; *D*, product obtained from *X* by hydrolysis, periodate oxidation, and methyl glycosidation.

The foregoing evidence indicates that the fucogalactan of *Ampullarius* sp. [branching points (29%) at C-3 and C-6 of the D-galactose residues] contains D-galactose (27%) and L-fucose (2%) as non-reducing end-groups and a preponderance of (1→6) (26%) over (1→3) (5%) linkages. The typical feature of this polysaccharide is the presence of (1→2)-linked D-galactose residues (11%), which were not observed in polysaccharides isolated from egg masses and albumen glands of snails[4,7,8]. No differences in the optical rotations and methylation results (Table I) of the heteropolysaccharides isolated either from albumen glands or egg masses were observed, suggesting that the fucogalactan from egg masses may be synthesized in the albumen gland. Since no glycogen was found in freshly collected egg masses, it is possible that the fucogalactan might play a role in the nutrition of snail embryos.

EXPERIMENTAL

General. — 3,4,6-Tri-*O*-methyl-D-galactose, isolated from a mixture of the partially methylated sugars [t.l.c., solvents (*i*) and (*m*)], was converted into its methylated alditol derivative, and a solution in chloroform was injected into an

ECNSS-M column [column (*e*)] at 180° fitted in a combined gas chromatograph double-focussing mass spectrometer (JEOL JMS-D100) at 180°, with enricher at 250°, injection port at 200°, and a flow rate of helium of 50 ml/min. The mass spectra were recorded at an ionizing potential of 30 eV, ionizing current at 300 μamp., ion multiplier voltage control of 1.3 kV, and a chamber temperature of 150°. The mass range was 1–600, scan time 60 min, and the visigraph paper speed 2 cm/sec.

Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter at 25°. Electrophoresis was performed with a Perkin–Elmer 38 apparatus and 0.05M sodium borate buffer (pH 9.2) at 20 mamp and 150 V, and, on acetylated paper, with a Fanem apparatus according to the procedure of Dudman and Bishop[16]. I.r. spectra were determined for KBr discs with a Beckman IR-8 spectrophotometer. G.l.c. was performed on an F & M chromatograph Model 810 R-12 (flame ionization), using helium as carrier gas and (*a*) 15% of butane-1,4-diol succinate polyester on 80–100 mesh acid-washed Celite (column, 120 × 0.4 cm i.d.) at 175° (flow rate, 40 ml/min); (*b*) 10% of polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] on 80–100 mesh Celite (column, 120 × 0.4 cm.i.d.) at 185° (flow rate, 75 ml/min); (*c*) 14% of LAC-4R-886 on 80–100 mesh (DMSC) Chromosorb W (column, 100 × 0.4 cm, i.d.) at 160° (flow rate, 40 ml/min); (*d*) 1:1 of 10% of butane-1,4-diol succinate polyester and 10% of Apiezon M on silver coated 60–80 mesh Chromosorb W (column, 120 × 0.4 cm i.d.) (Instrumentos Cientificos CG-São Paulo-Brazil) at 175° (flow rate, 75 ml/min); (*e*) 3% of ECNSS-M on 100–120 mesh Gas Chrom Q (column, 120 × 0.15 cm i.d.)[17] at 140–180° (flow rate, 10 ml/min). Retention times (*T*) are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside. Column (*c*) was used for quantitative analysis of the methylated sugars by the triangulation procedure[18]. Column (*d*) was used to determine the sugars as their alditol acetates[19], and the *T* values are related to that of D-arabinitol penta-acetate. Column (*e*) was used for quantitative analysis of the alditol acetates, with temperature programming (140–180°; 10°/min). The *T* values of partially methylated sugars (except 3,4,6-tri-*O*-methyl-D-galactose) were also compared with pure samples prepared by Duarte and Jones[11], and they agree well with those obtained by Aspinall[20] and Stephen[18] (Table I). Paper chromatography (p.c.) was performed by the descending method with Whatman Nos. 1, 4, and 3MM papers, using (*f*) 1:5:3:3 (upper layer) benzene–1-butanol–pyridine–water, (*g*) 8:2:1 ethyl acetate–pyridine–water, (*h*) 9:2:2 ethyl acetate–pyridine–water, and (*i*) 200:17:1 butanone–water–ammonia, and detection with either alkaline silver nitrate[21] or *p*-anisidine hydrochloride[22]. Mobilities are given as R_{GAL} (free sugars) and R_G values (methylated sugars); the latter are relative to 2,3,4,6-tetra-*O*-methyl-D-glucose. T.l.c. was performed on silica gel (Merck) with (*j*) 9:1 benzene–methanol, and (*k*) 200:47:15:1 benzene–ethanol–water–acetic acid, and detection with 5% ethanolic sulphuric acid at 150°. The mobility of alditol acetates is expressed relative to that of D-arabinitol penta-acetate.

Acetylation of the sugars was performed using either acetic anhydride–pyridine (1:1.3) for 4 h at 100°, or acetic anhydride–70% perchloric acid (14:0.1) for 2 h at 35°. The reactions were monitored by t.l.c. [solvents (*j*) and (*k*)]. Total sugar and protein

were determined by the phenol–sulphuric acid[23] and Lowry[24] methods, respectively.

Isolation and purification of the polysaccharide from egg masses. — Snails, *Ampullarius* sp., were collected in Morretes, Paraná (Brazil), and kept in well-aerated water. Egg masses (110) were collected from the walls of glass aquaria (6–12 h after egg laying), and homogenized in a blender with acetone. The solids were collected by filtration and exhaustively extracted (Soxhlet) with a hot 2:1 chloroform–methanol mixture for 24 h and with butanol saturated with water for 12 h. The residue was collected by filtration and thrice washed with acetone. The dry, extracted powder (21 g) was suspended in 0.1M ammonium acetate (600 ml, pH 8.5), and crystalline subtilisin (2 × 150 mg, Type VII, Sigma Chemical Co.) was added. The mixture was kept for 72 h at 37° with toluene as preservative. The polysaccharide solution was centrifuged, and the supernatant solution was dialyzed against tap water for 24 h, centrifuged, and deproteinized (6×) by the method of Sevag[25]. The aqueous phase after application of the Sevag procedure was then lyophilized. The dry residue (2.5 g) was dissolved in water and fractionated by the addition[11] of cetyltrimethylammonium bromide. The product (1.3 g, 6.3%) had $[\alpha]_D^{25}$ +51° (*c* 0.74, water). The purified polysaccharide (3 mg) was homogeneous on Sephadex G-200 (column, 80 × 2 cm; 8 ml/h), 2% Agarose (column, 90 × 1.5 cm; 3 ml/h), 4% Agarose (column, 90 × 2 cm; 4 ml/h), and Sepharose 6B-100 (Sigma Chemical Co.) (column, 97 × 1.7 cm; 12 ml/h). The yield of polysaccharide (after these chromatographic processes) was ~95% when tested by the phenol–sulphuric acid method. The products of acid hydrolysis of the polysaccharide had $[\alpha]_D^{25}$ +80° (*c* 1, water), and p.c. and g.l.c. showed the presence of D-galactose (*T* 3.27, column (*d*)] and L-fucose [R_{GAL} 1.7, *T* 0.64, column (*d*)]. The D-galactose, after crystallisation from ethanol, had $[\alpha]_D^{25}$ +80.5° (*c* 1, water).

Periodate oxidation of the polysaccharide. — A sample of the polysaccharide (50 mg) was oxidized in 0.01M sodium metaperiodate (100 ml) in the dark for 94 h at 0–2°. Aliquots (5 ml) of this solution were analysed for periodate uptake[26] and yield of formic acid[27,28]. After 94 h, one mole of polysaccharide had consumed 1.10 mole of periodate, and liberated 0.47 mole of formic acid, per mole of anhydro sugar.

Smith degradation of the polysaccharide[29,30]. — The polysaccharide (50 mg) was oxidized with 0.01M sodium metaperiodate (100 ml) for 94 h at 0–2°. Excess of oxidant was then destroyed with ethylene glycol (1 ml), and the solution was dialysed against tap water for 24 h. Sodium borohydride (50 mg) was added, and after 24 h, the excess of reductant was destroyed with 2M acetic acid. The solution was hydrolysed with M sulphuric acid for 5 h at 100°, and the hydrolysate was neutralized with barium carbonate (to pH 4.5). The products were treated with sodium borohydride (2 × 50 mg for 12 h). After removal of cations with Dowex X-8 (H^+) resin (200–400 mesh), borate ions were eliminated by successive treatments with methanol and evaporation. The product of Smith degradation, when analysed by p.c. and g.l.c. [alditol acetates, columns (*d*) and (*e*)], showed glycerol (72 μmole%), galactitol (28 μmole%), and traces of a compound (not quantitatively determined) which was believed to be propane-1,2-diol (from L-fucose).

Methylation of the polysaccharide. — The polysaccharide (1 g) was methylated four times by the Haworth procedure[31]. The reaction mixture was neutralized (dilute sulphuric acid) and salts were removed by dialysis against tap water. The syrupy product was methylated (4×) using tetrahydrofuran–methyl sulphate–sodium hydroxide[32,33]. As the product (0.85 g) still contained OH groups (i.r.), it was twice methylated by the Sandford and Conrad procedure[34] (yield, 0.80 g) and then by the Purdie method[35] (yield, 0.50 g). A solution of the methylated polysaccharide in chloroform was treated with light petroleum (b.p. 30–60°) and centrifuged. The dry polysaccharide, $[\alpha]_D^{25}$ −10° (*c* 1, chloroform), showed no i.r. absorption for hydroxyl.

Methanolysis of the methylated polysaccharide. — The methylated polymer (100 mg) was treated with 6% methanolic chloride (10 ml) for 6 h at 100° in a sealed tube. The cooled solution was neutralized with silver carbonate, and the mixture of methyl glycosides was analysed by g.l.c. [column (*c*)]. The *T* values of the products are given in Table I.

A methyl galactoside (*X*, 10 mg) with *T* 5.62 (Table II) was isolated from the foregoing mixture by t.l.c. [solvent (*k*)], and treated with 0.5M HCl (2 ml) for 5 h at 100°. The cooled solution was neutralized with silver carbonate[36], filtered, and lyophilized. The methylated reducing sugar was treated with sodium borohydride, and the product was acetylated (acetic anhydride–pyridine)[37]. The resulting methylated alditol derivative had *T* 2.48 [g.l.c., column (*e*)][38] relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. For similar conditions, Björndal *et al.*[38] reported *T* 2.50 for 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol.

Periodate oxidation of the methylated reducing sugars. — A sample of the methylated polysaccharide (10 mg) was methanolysed and the resulting glycosides were hydrolysed[36] with 0.5M HCl (2 ml) for 5 h at 100°. The resulting methylated reducing sugars were treated with 0.05M sodium metaperiodate (2 ml) in the dark for 2 h at 18°. Excess of oxidant was then destroyed by *myo*-inositol (10 mg), the solution was lyophilized, and the dry residue was extracted with chloroform. A sample of *X* described above was also treated with periodate. G.l.c. showed that only 3,4,6-tri-*O*-methyl-D-galactose (*T* 5.62, column (*c*), Table II] was oxidized and gave 2,3,5-tri-*O*-methyl-D-lyxose, the methyl glycoside of which had *T* 0.68 [column (*c*), Table II] and the alditol acetate *T* 0.48 [column (*e*), relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol]. Methyl 2,3,5-tri-*O*-methyl-D-lyxoside[39], $[\alpha]_D^{25}$ +46° (*c* 0.5, methanol) [+32° (*c* 1, water) on hydrolysis with 0.1M HCl for 50 min at 95°], had *T* 0.68 [column (*c*)]. The derived alditol acetate had *T* 0.48 [column (*e*)]; Björndal *et al.*[38] recorded *T* 0.48 [column (*e*)] for 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-D-lyxitol.

Isolation and purification of the polysaccharide from albumen glands. — A heteropolysaccharide (5.1%), isolated from albumen glands of *Ampullarius* sp. in the same way as described for the egg masses, had $[\alpha]_D^{25}$ +54° (*c* 1, water), and on hydrolysis gave D-galactose, m.p. 160°, $[\alpha]_D^{25}$ +80.5° (*c* 1, water) (derived mucic acid, m.p. 219°), and L-fucose. The polysaccharide was methylated as described above, and the product, $[\alpha]_D^{25}$ −6° (*c* 1, chloroform), was methanolyzed, and the mixture of methyl glycosides was analysed by g.l.c. [column (*c*), Table I]. No difference was

observed in the *T* values [columns (*a*) and (*b*)] of the methyl glycosides obtained from the polymers from albumen glands and egg masses.

Enzymic determination of L-*fucose.* — The L-fucose isolated by p.c. [solvent (*f*)] from the acidic hydrolysate (0.5M H_2SO_4 for 3 h at 100°) of the polysaccharide was assayed by L-fucose dehydrogenase of *Pullularia pullulans*[40]. The enzymic analysis showed that the fucose from each polysaccharide belongs to the L series.

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