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Note

L-Galactose in sulfated polysaccharides from tunicates

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We recently isolated and characterized novel sulfated polysaccharides from the connective tissues of invertebrates[1–4]. In extracts from the tunic of ascidians (Chordata, Tunicata), these polymers occur as different fractions that are distinct in molecular weight and chemical composition[1–3]. The high-molecular-weight fraction contains a high proportion of galactose, and comprises most of the sulfated polysaccharides extracted from the tunic of various species of ascidians.

We showed in a recent publication[3] that the galactose from the sulfated polysaccharides of the species *Styela plicata* occurs entirely in the L-enantiomeric form. In the present study, the same observation is extended to other species of ascidians. All results show that the sulfated glycans of the ascidian tunics are unique among known polysaccharides, in that their major constituent sugar is L-galactose, lacking entirely the D enantiomorph of this sugar.

EXPERIMENTAL

Extraction, purification, and chemical analysis of the high-molecular-weight polysaccharides. — The following species of ascidians were collected from Urca, Guanabara Bay, Rio de Janeiro, and identified as being *Styela plicata, Ascidia nigra, Herdmania monus, Clavelina* sp., and *Botryllus* sp. The polysaccharides were extracted, fractionated, and checked for purity as previously described[2,3] for *S. plicata*. The sugar composition and sulfate content were determined as previously described[2,3] for *S. plicata*. Optical rotation measurements were made with a digital polarimeter (Perkin–Elmer model 243-B).

Methylation of the ascidian polysaccharides. — Fraction F-1 (10 mg) of the ascidian polysaccharides was methylated by the Hakomori method[5], with the modifications introduced by Conrad[6]. The methylated polysaccharides were hydrolyzed with 4.0M trifluoroacetic acid for 6 h at 100°, the sugars reduced with borohydride, the alditols acetylated[7], and the acetates dissolved in chloroform and analyzed in a

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Hewlett–Packard g.l.c./m.s. unit, model 5987-A. Injection was made in the splitless mode on an SE-54 capillary column (25 m × 0.3 mm). The column was programmed to run for 2 min at 50°, then the temperature was raised to 170° at 60°/min, and held there for 45 min. The carrier gas was helium with a linear velocity of 20 cm/s.

^{13}C-N.m.r. spectra. — ^{13}C-N.m.r. spectra were recorded, with D_2O as the solvent (following prior H–D exchange), at 60°, with a Bruker WH-400 spectrometer. The chemical shifts (δ) were measured with respect to internal 1,4-dioxane (66.5 p.p.m. relative to tetramethylsilane).

Mild hydrolysis with acid. — This experiment was performed in order to examine the preponderance of either galactopyranosyl or galactofuranosyl units in the ascidian polysaccharides. A portion (~2 mg) of the ascidian polysaccharides was different times, mixed with 0.1 mL of ice-cold 0.2M NaOH, and the amounts of reducing sugars in the solutions measured by the Park–Johnson method[8]. These experiments included, as appropriate controls, the mild acid hydrolysis of *Helix pomatia* galactan, composed of galactopyranosyl units[9], and of a polysaccharide from *A. niger* rich in galactofuranosyl groups at nonreducing ends[10].

Preparation of acetylated 2-octyl glycosides. — The analysis of the galactosides formed from (+)-2-octanol was employed in order to assess the absolute configuration of the galactose in the ascidian polysaccharides, as previously described[11]. Solutions of the ascidian polysaccharides (2 mg) in 0.5 mL of 4.0M trifluoroacetic acid (TFA) were heated for 4 h at 100°, cooled, the acid evaporated in a rotary evaporator, 0.5 mL of (+)-2-octanol (Aldrich Chemical Co., Milwaukee, WI, USA) and a drop of TFA added to each tube, and these heated for 24 h at 130°. The solutions were then evaporated to dryness in a rotary evaporator, and the residue acetylated with 1:1 (v/v) acetic anhydride–pyridine. The acetylated 2-octyl glycosides were analyzed by gas–liquid chromatography with an OV-1701 capillary column run isothermally at 230°. Appropriate controls of acetylated (+)-2-octyl D- and L-galactoside were analyzed under the same conditions.

Isolation of galactose from the ascidian polysaccharides. — The sulfated polysaccharides (~10 mg) from each species of ascidian were hydrolyzed with 4.0M TFA for 5 h at 100°, cooled, the acid evaporated in a rotary evaporator, and the mixtures applied to Whatman paper No. 3MM, and subjected to descending chromatography in 3:2:1 (v/v) 1-butanol–pyridine–water for 36 h. A guide strip of the chromatogram was stained with silver nitrate, and the region corresponding to galactose was eluted with distilled water. Each eluate was concentrated to ~1 mL, and the concentration of hexose was measured by the Dubois reaction[12]. The specific rotation was measured with a digital polarimeter.

Enzymic incubations. — (*a*) *Oxidation with D-galactose oxidase.* Various amounts of the galactose isolated from the ascidian polysaccharides, as well as of standard D- and L-galactose, were incubated with 2 units of D-galactose oxidase[13], peroxidase (4 units), and the chromogen *o*-tolidine (0.1 mg) in 0.5 mL of 0.05M sodium acetate buffer (pH 7.0). The reaction mixtures were incubated at 37°, and the absorbance at 425 nm was measured periodically.

(*b*) *Oxidation with* L-*fucose dehydrogenase*. The galactose (~100 μg) isolated from the ascidian polysaccharides, as well as standard D- and L-galactose, were incubated with 0.2 unit of porcine liver L-fucose dehydrogenase[14] and 2.5 μmol of NAD^+ in 2 mL of 0.01M glycine–NaOH buffer (pH 8.0). The reaction mixtures were incubated for different times at 37°, and the formation of NADH was monitored by measurement of the absorbance at 340 nm.

RESULTS AND DISCUSSION

Table I shows the chemical analysis and the optical rotation of the high-molecular-weight fractions (F-1) of the sulfated polysaccharides extracted from the tunics of various species of ascidians. Fraction F-1 from all species has a sulfuric ester group, a high content of galactose, and a strong negative optical rotation. Small proportions of hexosamine are present in F-1 from *A. nigra*, *Botryllus*, and *S. plicata*, and small percentages of fucose and mannose are present in the polysaccharides from *Clavelina* and *A. nigra*, respectively. Furthermore, small proportions of glucose are detected in fraction F-1 from all species except *H. monus*.

The strong negative specific rotation (−100° to −119°) of fraction F-1 from the various species of ascidians is especially noteworthy. As these polysaccharides consist largely of galactose, their optical activities are compatible with two possible types of residue, namely, β-D-galactofuranosyl and α-L-galactopyranosyl. The specific rotations of methyl β-D-galactofuranoside and methyl α-L-galactopyranoside are −112° and −179°, respectively.

However, the former possibility is inconsistent with the fact that the ^{13}C nuclei of the various samples of F-1 are far more strongly shielded than are the corresponding nuclei of methyl β-D-galactofuranoside (see Table II), whereas they resonate in the range expected for methyl α-L(or D)-galactopyranoside. Furthermore, partial hydrolysis with 0.01M HCl for 4 h at 100° releases only 5%, or less,

TABLE I

CHEMICAL COMPOSITION AND SPECIFIC OPTICAL ROTATION OF FRACTION F-1 FROM DIFFERENT SPECIES OF ASCIDIANS

Species	*Molar ratio*						$[\alpha]_D^{20}$ *(degrees)*
	Gal	*Glc*	*Man*	*Fuc*	$HexNH_2$	*Sulfate/ total sugar*	
S. plicata	0.82	0.14	<0.01	<0.01	0.04	0.66	−119
A. nigra	0.67	0.13	0.09	<0.01	0.11[a]	0.17	−100
Botryllus	0.67	0.17	<0.01	<0.01	0.16[a]	0.53	−100
Clavelina	0.72	0.23	<0.01	0.05	<0.01	0.38	−104
H. monus	1.00	<0.01	<0.01	<0.01	<0.01	0.40	−110

[a]About 70% of the hexosamine is galactosamine.

TABLE II

^{13}C CHEMICAL SHIFT (δ) DATA FOR FRACTION F-1 FROM DIFFERENT SPECIES OF TUNICATES AND FOR STANDARD METHYL GALACTOSIDES

Sample	*C-1*	*C-2 to C-5*	*C-6*
F-1 from *S. plicata*	101.3–97.9	72.4–68.4[a]	62.4
F-1 from *A. nigra*	103.7–98.7	72.0–68.2[a]	62.4
F-1 from *Clavelina*	101.3	72.6–68.6[a]	60.7
α-Galactopyranose[19,20]	100.1	71.6–69.2	62.2
β-Galactofuranose[21]	109.9	84.7–71.7	63.6

[a]Not including the signals attributable to glycosidically linked or sulfated secondary carbon atoms, which resonate at 80.2–74.3 p.p.m.

of the total sugars found in these polysaccharides, as expected for a polysaccharide composed mainly of galactopyranosyl units.

Other evidence for the presence of galactopyranosyl (rather than galactofuranosyl) units in the ascidian polysaccharides was obtained by methylation studies. Fraction F-1 from *S. plicata* and *A. nigra* produces ~20% of 2,3,4,6-tetra-*O*-methylgalactitol and 45% of 2,6-di-*O*-methylgalactitol. Smaller proportions of 2,3,4-tri-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and 6-mono-*O*-methyl-galactitol are also formed. However, no methyl ether substituted on O-5 was found in the methylated ascidian polysaccharides.

The presence of α-L-galactopyranosyl units in these polysaccharides was unequivocally demonstrated by the finding that the F-1 from the various species of ascidians contains only L-galactose, and lacks its D-enantiomorph, as shown by the following observations. The specific rotations of the galactose isolated from fraction F-1 of *A. nigra, Botryllus, Clavelina, S. plicata,* and *H. monus* are $-53°$, $-47°$, $-71°$, $-79°$, and $-52°$, respectively. Comparison with the specific rotation of $-80°$ recorded for a mutarotated solution of authentic L-galactose, confirms the preponderance of the L-enantiomer of this sugar in the ascidian polysaccharides. The observation that the optical rotation of some isolated galactoses is not as negative as a mutarotated solution of standard L-galactose may be attributed to a small contamination with D-glucose, or to the presence of a small proportion of D-galactose.

A number of experiments indicated that D-galactose is not present. The absolute configuration of the galactose in the ascidian polysaccharides was confirmed by gas–liquid chromatographic analysis of the glycosides formed from a chiral alcohol[11]. The acetylated 2-octyl galactosides obtained from the ascidian polysaccharides show the same retention times as the glycosides from L-galactose, and no peaks were observed in the region of acetylated 2-octyl D-galactosides.

Furthermore, incubation with D-galactose oxidase (see Fig. 1A) showed that the galactoses isolated are resistant to this enzyme, and it also excluded the presence of some D-galactose. The fact that no D-galactose oxidation product was detected colorimetrically (which should be feasible at a level of 1 μg), clearly demonstrated that the galactose produced by acid hydrolysis of the ascidian poly-

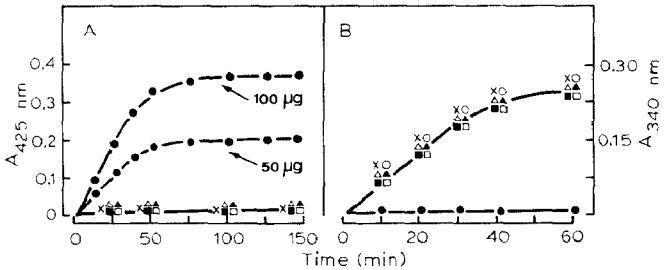

Fig. 1. Oxidation of the galactose obtained from the hydrolyzates of the polysaccharides from the various species of ascidians by D-galactose oxidase and L-fucose dehydrogenase. A. The rate of oxidation by D-galactose oxidase. The incubation mixtures contained 50 µg and 100 µg of standard D-galactose (—●—), or 100 µg of the galactose obtained from *S. plicata* (—□—), *A. nigra* (—■—), *H. monus* (—△—), *Clavelina* sp. (—▲—), and *Botryllus* sp. (—×—). B. The oxidation of galactose by L-fucose dehydrogenase. The incubation mixtures contained 100 µg of standard L-galactose (—○—), 100 µg of standard D-galactose (—●—), or the galactose obtained from the various species of ascidians (same symbols as already described). The incubation conditions are described in the Experimental section.

saccharides is exclusively the L isomer. In addition, the galactoses isolated are oxidized by L-fucose dehydrogenase (see Fig. 1B). Their rates of oxidation are approximately the same as the rate for authentic L-galactose (see Fig. 1B), an additional indication that the galactose isolated from the ascidian polysaccharides occurs as the L isomer.

The resistance of the chemically desulfated fraction F-1 from *S. plicata* to the action of α- or β-D-galactosidase, demonstrated in our previous study[3], is another indication of the absence of D-galactose from these polysaccharides.

Small proportions of L-galactose have been reported to be present in seaweed carrageenans[15]; however, these residues were always accompanied by high percentages of D-galactose. Two other polysaccharides that contain L-galactose are not sulfated. In snail galactans[9,16,17], the L-galactose is accompanied by at least three times as much D-galactose, whereas, in flax-seed mucilage[18], it occurs together with D-galacturonic acid, L-rhamnose, and D-xylose. The ascidian polysaccharides are unique among these previously described polysaccharides, in that their major constituent is L-galactose, the D enantiomorph being entirely absent.

The biological relevance of this finding is still unclear. Possibly, the presence of L isomers of galactose makes these polysaccharides resistant to degradation by D-galactosidases, and therefore avoids digestion of the ascidian tunics by the microorganisms present in sea water.

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