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Note

Galactan sulfate from the test of tunicates

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It has long been known that the test of tunicates contains a cellulose-like polysaccharide named tunicin[1]. Recently, in our laboratory, considerable proportions of water-soluble polysaccharides were found in the test of the tunicate *Halocynthia roretzi*, and, after ethanol fractionation, a chitin sulfate-like polysaccharide was isolated[2].

In this paper, a galactan sulfate was isolated from the test of the tunicates *H. roretzi* and *Styela plicata*. It gave a single band on cellulose acetate electrophoresis and was composed of equimolar amounts of galactose and sulfate. The i.r. spectrum indicated that it contains both equatorial and axial sulfate groups. Its optical rotation was strongly negative. After complete hydrolysis by acid, it gave L-galactose but no D-galactose. Galactose 4-sulfate and (presumably) galactose 6- (or 3-) sulfate were obtained after gel chromatography and preparative electrophoresis of the mild-acid hydrolyzate. The foregoing data suggest that the preponderant structure of the galactan sulfate is an α-linked L-galactan sulfate composed of L-galactose 4-sulfate and L-galactose 6- (or 3-) sulfate residues.

Several kinds of galactan sulfates have been isolated from various species of seaweeds[3]. However, their occurrence in the animal kingdom has been reported only in the jelly coat of eggs of the sea urchin, *Echinus esculentus*[4]. L-Galactose has been found in the water-soluble polysaccharides of the red seaweeds[5–8], together with D-galactose and 3,6-anhydro-L-galactose. In the animal kingdom, the galactan sulfate from *E. esculentus* and the galactan from the snail *Helix pomatia*[9], have been reported to contain L-galactose. A galactan sulfate composed of residues of L-galactose only, described in this paper, is an extremely rare instance in Nature. The isolation of L-galactose 4-sulfate seems to be the first example from natural sources, and the synthesis of this sugar sulfate has not yet been reported.

EXPERIMENTAL

Isolation. — The water-soluble polysaccharides were extracted from the test of the tunicates, *H. roretzi* and *S. plicata*, by exhaustive pronase digestion, and the

TABLE I

ANALYSES OF FR. 45 FROM *H. roretzi* AND FR. 35 FROM *S. plicata*

Component	*Fr. 45*		*Fr. 35*	
	Molar ratio	(%)	*Molar ratio*	(%)
Hexose (as galactose)	1.00	45.3	1.00	56.9
Glucose/galactose	0.13		0.13	
Sulfate	0.98	23.9	0.81	24.9
Hexosamine		1.0		1.8
Uronic acid		1.0		2.0
Lowry protein		4.2		3.2
3,6-Anhydrogalactose		0.5		0.4
$[\alpha]_D$	—170° (0.22, H_2O)		—149° (0.23, H_2O)	

polysaccharides were fractionated with ethanol as described previously[2]. At 25, 45, and 75% concentrations of ethanol, the polysaccharides from *H. roretzi* were precipitated in yields of 5, 45, and 43%, respectively, whereas the polysaccharides from *S. plicata* were precipitated at 20, 35, and 66% ethanol concentrations and the yields were 5, 57, and 31%, respectively.

Characterization. — The 45% ethanol fraction (Fr. 45) from *H. roretzi*, and the 35% ethanol fraction (Fr. 35) from *S. plicata*, gave a single band showing the same mobility on cellulose acetate electrophoresis in the four kinds of buffer: 0.2M calcium acetate[10], 0.1M barium acetate[11], 0.1M hydrochloric acid[12], and 0.05M acetic acid–pyridine[13], pH 6.0. The analytical data for these fractions are shown in Table I. The general analyses were carried out as described previously[2]. The molar ratio of sulfate to galactose was about 1 : 1. Both fractions contained small proportions of glucose and peptide, and the molar ratio of glucose to galactose by g.l.c.[14] was 0.13 : 1. The contents of hexosamine and uronic acid were very low. 3,6-Anhydrogalactose[15] was negligible before and after alkaline treatment[16]. The optical rotations of both fractions showed strongly negative values. The i.r. spectra showed a strong absorption band in the 820-cm^{-1} region and a weak absorption band in the 850-cm^{-1} region. From the foregoing results, Fr. 45 and Fr. 35 were considered to be galactan sulfates consisting mainly of galactose monosulfate.

Structural analysis. — Fr. 45 (50 mg) was hydrolyzed in 0.1M hydrochloric acid (2 ml) for 1 h at 100°. The hydrolyzate was neutralized with sodium hydroxide solution and fractionated by gel chromatography on a column (2.3 × 145 cm) of Sephadex G-15. The water eluates, analyzed by the anthrone reaction, showed three peaks. The second peak had the same retention volume as that of glucose 6-sulfate, and the fraction obtained from this peak was purified by preparative, paper electrophoresis[17] in 0.05M acetic acid–pyridine, pH 6.0. The region corresponding to glucose 6-sulfate was further fractionated by electrophoresis[18] in 0.1M borate buffer, pH 10.0. Two main bands (Frs. A and B), whose M_G values were 1.03 and 1.30, were

eluted with water, and passed through a column of Dowex-50 (H^+) resin. The effluent was evaporated, *in vacuo*, with repeated addition of methanol, and freeze-dried.

The ratio of sulfate to galactose of Fr. A was 1.0 and the degree of polymerization, as determined by the method of Timell[19], was 1.0. The M_G value of Fr. A corresponded to that of galactose 4- (ref. 20) or 2-sulfate[21]. The i.r. spectrum showed an absorption band at 850 cm^{-1}. On periodate oxidation[22], Fr. A consumed the following mol of oxidant per mol (time in h): 0.84 (0.5), 1.29 (1), 1.36 (2), 1.60 (3.5), 2.22 (46.5), and 2.55 (142.5). These data suggest that Fr. A is galactose 4-sulfate or 2-sulfate[20,21]. Fr. A gave a red color with triphenyltetrazolium chloride[23], which means that Fr. A has no substituent[24,25] on C-2, and gave a blue-grey color with diphenylamine–aniline, characteristic of galactose 4-sulfate[21]. From these results, Fr. A is considered to be galactose 4-sulfate.

The ratio of sulfate to galactose of Fr. B was 1.0 and the degree of polymerization was 1.0. The M_G value of Fr. B corresponded to that of galactose 6- or 3-sulfate[26]. The i.r. spectrum showed a strong absorption band at 820 cm^{-1}. The amount of Fr. B was not enough for further examination, and we could not determine whether it was galactose 6-sulfate or 3-sulfate.

The hydrolyzate of Fr. 35 gave the same analytical results as those from Fr. 45, except that the ratio of Fr. A to Fr. B was somewhat different.

Each galactan sulfate was hydrolyzed in M hydrochloric acid for 3 h at 100° and passed through a column of Amberlite MB-3, and the neutral-sugar fraction was obtained. The neutral-sugar fraction gave no color in an enzymic assay that used D-galactose oxidase and peroxidase[27], showing that there was no D-galactose present. With glucostat, the D-glucose content in the fraction was estimated to be 12%; this is consistent with the result obtained by g.l.c.[14]. Although the optical rotation of the neutral-sugar fraction was −64.6° (*c* 0.65, water), after correction for the D-glucose content, the optical rotation of galactose became −80.4° (*c* 0.57, water) in agreement with the published constant[28]. These results indicated that the component galactose is of the L configuration.

The treatment of galactan sulfate fractions with 0.4M sodium hydroxide in the presence of 0.3M sodium borohydride and 0.01M palladium chloride for 24 h at 30° resulted in the decomposition of 43% of the serine and 53% of the threonine in Fr. 45, and 54% of the serine and 62% of the threonine in Fr. 35, an increase of alanine and the formation of 2-aminobutanoic acid. These results suggest that the linkage between galactan sulfate and peptide consists of O–glycosyl bonds to serine and threonine.

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