ISOLATION OF THE GALACTAN OF *Phyllophora nervosa* AND CHARACTERIZATION OF ITS STRUCTURE

M. S. Dudkin and E. I. Kozarev

UDC 549.917+639.64

The structure of sulfated galactans used in the food and textile industries has been studied comparatively little [1-3].

We have investigated the structure of a water-soluble sulfated galactan from the red alga *Phyllophora nervosa* collected in the Black Sea in August, 1969. The polysaccharide was isolated by extracting the alga with hot water followed by the treatment of the extract with "Cetavlon." Two fractions were isolated — I, neutral, and II, acidic. Their chemical compositions are:

	I	*II*
$[\alpha]_D^{20}$, deg	−53.7	+38.46
SO_4, %	—	18.3
3,6-Anhydrogalactose, %	0.12	22.50
Total neutral monosaccharides, %	51.99	52.14
Composition of hydrolyzate, %		
galactose	15.18	52.14
glucose	17.89	—
arabinose	5.84	—
xylose	13.08	Traces
fucose	+	—
ribose	+	—

Fraction II was characterized by the presence basically of a single monosaccharide — galactose — which made its further study desirable. For further purification, fraction II was reprecipitated with ethanol and subjected to gel filtration on Sephadex G-75 until its monosaccharide content was constant. The homogeneity of the polysaccharide was shown in parallel by electrophoresis in buffer systems with various pH values. It can be seen from the facts given above that the galactan studied is sulfated and contains 3,6-anhydrogalactose residues.

To determine the structure of the galactan we used periodate oxidation, methylation, complete and partial acid methanolysis, and desulfation.

Attempts to perform the oxidation of the galactan of *Phyllophora nervosa* with $NaIO_4$ of various concentrations did not lead to positive results, which shows the absence of free vicinal hydroxy groups.

The desulfated galactan was methylated and subjected to methanolysis, giving the dimethyl acetal of 2-O-methyl-3,6-anhydro-D-galactose, 2,3,4,6-tetra-O-methyl-D-galactose, and 2,4,6-tri-O-methyl-D-galactose. Thus, it was shown that the polysaccharide is constructed of galactose residues connected to one another by 1 → 3 glucosidic bonds and of 3,6-anhydrogalactose residues connected by 1 → 4 bonds.

The nature of the bond between the sulfuric acid and galactose residues in the initial galactan was determined. For this purpose the galactan was subjected to complete methanolysis. The methanolysis products were separated and identified in the following way (the results are given in Table 1).

M. V. Lomonosov Odessa Technological Institute of the Food Industry. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 693-698, November-December, 1976. Original article submitted June 20, 1976.

TABLE 1. Isolation and Identification of the Methanolysis Products of the Galactan

Fraction	Solvent	$[\alpha]_D^{20}$, deg		Melting point, °C		Substance
		found	literature data	found	literature data	
Chromatography of a chloroform extract						
I	Chloroform–acetone (1-8%)	±0	±0	Syrup	—	Dimethyl acetal of 2-O-methyl-3,6-anhydro-D-galactose [5]
II	Chloroform–acetone (25-30%)	+80,9	+80,0	139,5	140	Methyl 3,6-anhydro-α-D-galactopyranoside [5]
III	Chloroform–acetone (35-40%)	+32,9	+36,2	220	220	Dimethyl acetal of 3,6-anhydrogalactose [5]

By the crystallization of an aqueous solution we isolated methyl β-D-galactopyranoside, and by ion-exchange chromatography on Amberlite IR-4[b] we isolated barium methyl sulfate, $[\alpha]_D^{20} \pm 0$.

The results of methanolysis show that the sulfuric acid residues are bound to the galactose by an ester bond which is hydrolyzed by hydrogen chloride to methyl sulfate. The 3,6-anhydrogalactose residues in the structure of the agaroid are present in the D form, and 5.7% of the 3,6-anhydrogalactose is esterified with methanol at C_2.

To determine the positions of the sulfate groups in the galactan we studied the kinetics of the desulfation of the polysaccharide by hydrogen chloride in dry methanol. The desulfation constant at 22°C was $K_1 = 0.9771 \cdot 10^3$, and at 32° $K_2 = 1.1743 \cdot 10^3$.

The change in the order of the desulfation constant after treatment at 22°C for 18 h and at 32°C for 15 h shows a nonsynchronous desulfation process possibly caused by different stabilities of the sulfates at the primary and secondary carbon atoms of the monosaccharide unit of the polysaccharide. The desulfated polysaccharide contained D-galactose (68.94%), 3,6-anhydrogalactose (7.50%), and SO_4 (5.17%). The amounts of these substances in the initial galactan were 52.97, 22.50, and 18.30%, respectively.

The strongly alkaline reduction of the galactan of *Phyllophora nervosa* in the presence of $NaBH_4$ and NaOH gave a modified polysaccharide with a changed chemical composition: 39.80% of D-galactose, 35.59% of 3,6-anhydrogalactose, and 5.70% of SO_4.

The increase in the amount of 3,6-anhydrogalactose in the modified galactan is possibly due to the conversion of 6-O-sulfogalactose units into 3,6-anhydrogalactose units.

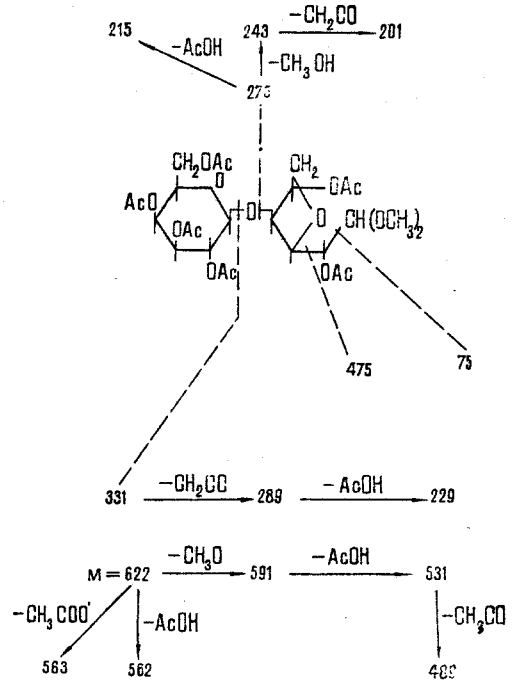
The IR spectra of the initial and the reduced galactans showed strong S=O vibrations in the 1250-cm^{-1} region – which is the characteristic peak of all sulfates. The initial galactan showed a peak at 816 cm^{-1} corresponding to sulfate groups on primary carbon atoms. This peak was not found in the reduced agaroid. A peak in the 920-cm^{-1} region corresponds to the vibration of the ring of the 3,6-anhydro system [1, 6, 7].

Partial hydrolysis of the galactan led to the complete splitting off of the sulfo groups and to the formation of an oligosaccharide which, after reduction with $NaBH_4$ and hydrolysis, gave D-galactose and 3,6-anhydrodulcitol in a ratio of 3:1.

After partial methanolysis and preparative chromatography on a carbon-Celite column, the dimethyl acetal of carrabiose was isolated on 3 MM paper. The nature of the oligosaccharide was established by methylation, by IR spectroscopy, and by mass spectrometry.

A mass-spectrometric investigation of the hexaacetate of the dimethyl acetal of carrabiose showed that the decomposition of the molecule under the action of electrons took place in the manner shown in the scheme on the next page.

A peak with m/e 622 corresponds to the molecular ion. A strong peak with m/e 72 shows the nature of the functional group at C_1. A fragment with a mass number m/e of 475 characterizes a 1 → 4 glycosidic bond between the galactose and 3,6-anhydrogalactose residues. The 3,6-anhydrogalactose residue is shown by a peak with m/e 275 [8]. In agreement with the results of measurements of the optical activities of the dimethyl acetal of carrabiose,



Fragmentation of hexa-O-acetylcarrabiose

$[\alpha]_D^{22.5}$ +25.9, and of its acetate, $[\alpha]_D^{22.5}$ −14.7 (literature data: $[\alpha]_D^{20}$ +24.6 and $[\alpha]_D^{20}$ −16.7° [11, 12]) the glycosidic bond of the monosaccharide residues has the β configuration. This was also confirmed by the IR spectra of these derivatives (868-880 cm^{-1}).

Thus, according to the results of periodate oxidation and of the chromatography of hydrolyzates of the methylated polysaccharide, the galactan molecule is not branched. The high contents of 2,4,6-trimethylgalactose and of the dimethyl acetal of 2-O-methyl-3,6-anhydrogalactose, and other information, show the presence of 1 → 3 bonds between the galactose residues and of 1 → 4 bonds between the 3,6-anhydrogalactose residues. Methanolysis of the initial sulfated galactan led to the formation of alkyl derivatives of galactose and of 3,6-anhydrogalactose in the D form, and the production of barium methyl sulfate shows the presence of an ester bond between the sulfuric acid and galactose residues, which was previously denied [3].

The results of partial methanolysis have shown the regularity of the galactan from the alga *Phyllophora nervosa*, which is characterized by a 4-O-β-D-galactosyl-3,6-anhydro-D-galactose unit.

EXPERIMENTAL

For chromatography we used type FN-3 paper and KSK silica gel, and for ion-exchange chromatography Amberlite IR-4[b] and KU-2. The solvent systems were: 1) butanol-1-ol—benzene—pyridine—water (5:1:3:3); 2) butanol—ethanol—water (4:1:2); 3) butan-1-ol—pyridine—water (6:4:3); 5) chloroform—acetone (1:1); 6) butanol—acetic acid—water (4:1:5); 7) water-saturated cyclohexanol; 8) chloroform—acetone (4:1); 9) toluene—ethanol (9:1).

Chromogenic agents: 1) aniline phthalate; 2) o-aminophenol phosphate; 3) a mixture of $AgNO_3$ and NH_4OH; 4) concentrated H_2SO_4. The 3,6-anhydrogalactose was determined by Japhe's method [9] and the sulfates by the gravimetric method in the form of $BaSO_4$ [10]. The IR spectra were taken on an IKS-14 instrument in tablets of KBr, and the mass spectra on an MKh-1303 instrument. The materials were introduced directly to the ion source at 70°C.

Isolation of the Galactan. The air-dry alga was comminuted and was treated successively with ether, acetone, and hot ethanol, and was dried and extracted with water at 90°C. The galactan was precipitated with "Cetavlon" [11] in the form of a complex from an extract decolorized with activated carbon. The complex was decomposed with 10% NaCl solution, dialyzed, and precipitated with ethanol.

Methylation of the Galactan. The galactan was desulfated with HCl in methanol at 15°C for 50 h. The desulfated polysaccharide was methylated by Haworth's method and methylation was brought to completion by Purdie's method [11]. The methylated galactan was passed through a column of Al_2O_3, being eluted with mixture 9. The eluate was evaporated in vacuum. The product contained 35.45% of CH_3O. The methylated galactan was subjected to methanolysis with 7.2% $HClO_4$, and the reaction mixture was neutralized with AV-17 anion-exchange resin and evaporated. The methyl glycosides were separated preparatively in a silica gel column using system 8. After rechromatography under the same conditions, three fractions were isolated. According to the results of chromatography on silica gel and paper with markers in systems 2 and 8, the dimethyl acetal of 2-O-methyl-3,6-anhydro-D-galactose, 2,3,4,6-tetra-O-methyl-D-galactose, and 2,4,6-tri-O-methyl-D-galactose were present. The latter two sugars were also identified as the anilides. Their melting points were, respectively, 189.2 and 170.2°C (literature data: 190 and 170°C [4]).

Methanolysis of the Initial Galactan. The galactan was heated with 3% HCl in dry methanol at 70°C for 20 h. The methanolyzate was neutralized with $Ba(OH)_2$ and passed through a column of IR-4^b anion-exchange resin and KU-2 cation-exchange resin. The deionized solution was evaporated in vacuum to a syrup $[\alpha]_D^{20}$ +64.20 (c 0.64); methanol). The dimethyl acetal of 2-O-methyl-3,6-anhydrogalactose, the dimethyl acetal of 3,6-anhydrogalactose, methyl 3,6-anhydrogalactoside, and methyl galactoside were identified by thin-layer chromatography on silica gel in systems 5 and 7. These substances were isolated by preparative chromatography on silica gel using gradient elution with mixtures of chloroform and acetone (1 → 50%). By washing the column of ion-exchange resin (after the deionization of the methanolyzate) with 2 N H_2SO_4, treatment with a hot solution of $Ba(OH)_2$, and separation of the precipitate we obtained the water-soluble barium methyl sulfate. The solution was evaporated and analyzed. Found: $[\alpha]_D^{20}$ ±0; and qualitative analysis showed the presence of Ba^{2+}, SO_4^{2-}, and carbon.

Partial Methanolysis. The polysaccharide was heated with 0.5% HCl in dry methanol at 70°C for two hours. The solution was neutralized with $Ba(OH)_2$ and passed successively through a column which contained IR-4^b cation-exchange resin and KU-2 and was concentrated in vacuum. Paper chromatography in system 2 and thin-layer chromatography on silica gel in systems 5 and 8 with markers showed the presence of methyl D-galactoside, the dimethyl acetal of carrabiose, and the dimethyl acetal of 3,6-anhydrogalactose.

The substances were separated on a column containing activated BAU carbon partially deactivated with 1% stearic acid and filled with Celite 545. The carrabiose derivative was rechromatographed on 3 MM paper in system 2. The compound obtained was identified in the form of the methyl and acetyl derivatives.

Methylation was performed by Hakomori's method [12]. The completely-methylated product was purified on silica gel in system 8.

Acetylation of Carrabiose. Carrabiose was acetylated with acetic anhydride in pyridine by the method described previously [13]. The melting point of the crystalline hexaacetate was 142.5°C, $[\alpha]_D^{22.5}$ −14.7.

Desulfation with HCl in Absolute Methanol. Weighed samples of the galactan were sealed in tubes with 0.1 N HCl in absolute methanol. After predetermined intervals of time, the tubes were opened, the methanol was removed by filtration, and the residue was washed with cold methanol until chloride ions were absent. The filtrates were combined and evaporated, and the sulfates in the residue were determined gravimetrically.

Desulfation by Strongly Alkaline Reduction. The galactan was dissolved in water and reduced with $NaBH_4$ at room temperature for 16 h, and then $NaBH_4$ and NaOH were added in portions over two hours with heating to 80°C as described previously [13]. After standing in the cold for 24 h, the solution was neutralized, deionized, and dialyzed. The reduced galactan was precipitated from the evaporated solution with ethanol. It was dried over P_2O_5.

SUMMARY

1. The chemical composition of the galactan of the Black-Sea alga *Phyllophora nervosa* has been established and its structure has been studied. The polysaccharide is linear and is constructed of D-galactose residued connected by 1 → 3 glycosidic bonds and of 3,6-anhydro-D-galactose residues connected by 1 → 4 bonds: —(4-O-β-D-Gal*p*-1→3-α-D-3,6-AhGal*p*-1)—.

2. The ester nature of the bond of the sulfuric acid residues with the primary and secondary carbon atoms of the galactose residues has been shown.

LITERATURE CITED

1. A. I. Usov, M. A. Rekhter, and N. K. Kochetkov, Zh. Obshch. Khim., 39, 905 (1969).
2. V. Gruner, Coloq. Internat. Centre Nat. Rech. Scient., 103, 183 (1961).
3. V. A. Evtushenko and M. A. Antonova, Zh. Prikl. Khim., 40, 1767 (1967).
4. J. K. N. Jones and G. H. Thomas, Can. J. Chem., 39, 197 (1961).
5. S. Hirase, K. Araki, and K. Watanabe, Bull. Chem. Soc. Jpn., 40, 1442 (1967).
6. J. R. Turvey, Adv. Carbohydr. Chem., 20, 183 (1965).
7. K. Nakanishi, Infrared Absorption Spectroscopy. Practical, Holden-Day, San Francisco (1962).
8. A. I. Usov, M. A. Rekhter, and N. K. Kochetkov, Zh. Obshch. Khim., 40, 2732 (1970).
9. W. Japhe, Anal. Chem., 32, 1327 (1960).
10. S. Peat, J. R. Turvey, and D. A. Rees, J. Chem. Soc., 1590 (1961).
11. N. K. Kochetkov, Methods of Carbohydrate Chemistry [in Russian], Moscow (1967).
12. S. Hakomori, J. Biochem., 55, 205 (1964).
13. D. A. Rees, J. Chem. Soc., 5158 (1961).

FRACTIONATION OF THE POLYSACCHARIDES OF *Chara aculeolata*

N. K. Cherno, M. S. Dudkin, and I. V. Areshidze

UDC 547.917

The structure of the polysaccharides of *Chara* algae has been little studied [1, 2]. Continuing investigations in this field, we have fractionated these polymers.

The polysaccharides were isolated by the successive extraction of the alga with various solvents, and their amounts in the raw material were determined. To characterize the products isolated we studied the monomeric compositions of hydrolyzates by various methods of chromatography, enzymatic hydrolysis, and spectrophotometry. In selecting the conditions for fractionation we started from the results obtained in a determination of the overall chemical composition of *Chara aculeolata* [3].

The scheme for fractionating the alga consisted of the following stages: isolation of the water-soluble substances, isolation of the substances soluble in ammonium oxalate, and isolation of the substances soluble in alkali.

The results of the fractionation are given in Tables 1-3. When the alga was treated with various solvents, more than 50% of the dry matter of the alga passed into solution, about 22% of it consisting of carbohydrate-containing polymers.

The amount of water-soluble polysaccharide (WSP) in the alga was 3.5%. In its hydrolyzate glucose predominated and the other monomers were present in practically equal amounts, with the exception of rhamnose (see Table 3).

The fractionation of the total WSPs on Sephadexes C-75, 100, and 150 showed that they contained two fractions with different molecular weights differing in their monomeric composition. The polymer with the lower molecular weight had a neutral character and that of higher molecular weight an acid character.

The neutral polysaccharide was a starch-like substance, as was shown by: a) a positive reaction with iodine; b) the specific nature of the UV spectrum of its iodine complex; and c) a high degree of attackability by amylase.

M. V. Lomonosov Odessa Technological Institute of the Food Industry. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 698-702, November-December, 1976. Original article submitted August 8, 1976.