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Production of Carbohydrate by the Marine Diatom *Chaetoceros affinis* var. *Willei* (Gran) Hustedt. III. Structural Studies of the Extracellular Polysaccharide

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Methylation and periodate oxidation studies have been carried out on the extracellular polysaccharide from cultures of *Chaetoceros affinis* var. *Willei* (Gran) Hustedt. It is apparent from the results obtained that the polysaccharide is highly branched. The results from periodate oxidation concomitant with the methylation studies indicate that the main part of the rhamnose is located in the outer part of the molecule, whereas the fucose and galactose are present both in the outer and inner parts. Apart from being present as end groups the rhamnose is 1,2-linked, galactose 1,4- and 1,3- (major) and some of the fucose 1,3-linked while the main part is present at branch points.

It is well known that many planktonic algae excrete considerable amounts of organic material into the surrounding medium, and that this material in many cases at least partly consists of carbohydrates. Very little is known about the nature of these carbohydrates. Recently, the production of soluble, extracellular polysaccharides has been reported from several diatom species. Allan *et al.*[1] studied eight species, and found significant amounts of soluble polysaccharide in the medium in all cases. The amounts were very small (less than 5 mg/l or 0.2 mg per 10^8 cells), with the exception of one species, *Nitzschia frustulum*, which in standard one-liter culture in enriched seawater produced 15.6 mg/l (1.3 mg per 10^8 cells) in 7 days, and in mass cultures over two weeks were reported to give as much as 150 mg/l (9 mg per 10^8 cells), depending upon conditions like salinity and nutrient levels. Hydrolysis of the polysaccharide gave rhamnose (24 %), mannose (34 %), galactose (8 %), and two unidentified components (14 and 20 %). The composition of the polysaccharide depended upon salinity.

In a study of *Chaetoceros affinis* (clone CH 1), Myklestad and Haug [2] reported the production of an extracellular polysaccharide in amounts of 16 – 40 mg/l (10 – 20 mg per 10^8 cells). The production took place in the stationary growth phase, after the main production of glucan was finished. Later studies [3] have shown that two other *Chaetoceros* species, *C. curvisetus* (CH 24) and *C. decipiens* (CH 40), also produce substantial amounts of extracellular polysaccharides, while in a number of other species, only very small amounts of extracellular polysaccharide can be detected. Both the amounts and the composition of the extracellular polysaccharide strongly suggest that in the case of the three *Chaetoceros* species mentioned above, the polysaccharide is excreted into the medium, and that it is not a case of leakage from dead or dying cells. The function of the polysaccharide in the life of the diatom is unknown. These extracellular polysaccharides all gave fucose, rhamnose, galactose, and sulfate on hydrolysis.

The present paper describes structural studies of the extracellular polysaccharide produced by *Chaetoceros affinis*.

RESULTS AND DISCUSSION

The cultivation of the diatom and isolation of the extracellular polysaccharide has been de-

Table 1. Composition of polysaccharide before and after periodate oxidation.

	Carbohydr. content [a] mg	Wt. ratio rha:fuc:gal	Am. of each sugar; mg (calculated)
Start. material	35.0	35:39:26	12.2:13.6:9.1
Prod. after 1st. periodate oxidation	15.8	12:64:24	1.9:10.1:3.8
Prod. after weak acid hydr.	13.1	7:68:25	0.9:9.0:3.3
Prod. after 2nd. periodate oxidation	8.5	4:75:21	0.3:6.4:1.8

[a] Determined by the phenol-sulfuric acid method.[5]

scribed previously.[2,4] The polysaccharide had $[\alpha]_D = -74°$, a carbohydrate content [5] of 80 %, $-SO_3Na$ was 8.9 % [6] corresponding to an equivalent weight of 1157. Potentiometric titration of the polysaccharide gave an equivalent weight of 850–900, which, if the only anion present is sulfate, was calculated to correspond to 11.4–12.1 % sulfate. The polysaccharide moved as a single, anionic compound in free-boundary electrophoreses both at pH 2 and pH 7, with approximately the same mobility at both pH values.[4]

Analysis for phosphate gave negative results, but small amounts of protein (1.5 % of the sample) were present.[4]

Complete acid hydrolysis of the polysaccharide and analysis by GLC of the derived alditol acetates [7] showed the presence of rhamnose, fucose, and galactose in the proportions 35:39:26 (Table 1). No other sugars were detected. The presence of small amounts of arabinose and traces of other sugars reported previously [4] was most probably due to contamination.

Periodate oxidation [8] was carried out as described under "Experimental". The polysaccharide reduced 0.59 mol of periodate per C_6-anhydro-unit (Table 2). Complete acid hydrolysis of the derived polyalcohol and analysis of the derived alditol acetates by GLC showed that the sugars rhamnose, fucose, and galactose were present in the polyalcohol in the proportions 12:64:24. From Table 1 it can be seen that the periodate cleaved 87.5 % rhamnose, 25.7 % fucose and 58.3 % galactose of the total of the respective sugars in the starting material.

After weak acid hydrolysis followed by dialysis and another periodate oxidation, the polymer reduced 0.36 mol of periodate per C_6-anhydro-unit (Table 2).

Table 1 shows that of the original carbohydrate content of the polysaccharide, 24 % carbohydrate still remains after the second periodate oxidation. This polyalcohol contains the sugars rhamnose, fucose, and galactose in the proportions 4:75:21, which is equivalent to 2.5, 47, and 19.8 % left of the respective sugars present in the starting material.

These results show that most of the rhamnose is vulnerable to periodate oxidation, and must be present mainly in the outer part of the

Table 2. Periodate consumption.

Periodate oxidation	Wt. carbohydr.[a] mg (mmol)	$NaIO_4$ added mg (mmol)	Volume acetate buffer pH 4 ml	Periodate consumption mol IO_4/C_6-anhydr.
1st. oxidation	35.0 (0.22)	106.5 (0.50)	10	0.59
2nd. oxidation	13.1 (0.08)	33.5 (0.16)	10	0.36

[a] Determined by the phenol-sulfuric acid method.[5]

Table 3. GLC analysis of the methylated alditol acetates.

	Retention time[a] Column 1	Column 2	Area of peaks % of total Column 2
2,3,4-Tri-*O*-methylrhamnose	0.42	0.46	12.8
2,3,4-Tri-*O*-methylfucose	0.64	0.59	4.4
3,4-Di-*O*-methylrhamnose	0.91	0.87	17.0
2,4-Di-*O*-methylrhamnose	[b]	0.93	4.3
2,4-Di-*O*-methylfucose	1.12	1.01	4.6
2,3,4,6-Tetra-*O*-methylgalactose	1.26	1.15	7.6
2-*O*-Methylrhamnose	1.55	1.35	1.2
2-*O*-Methylfucose	1.65	1.41	5.0
4-*O*-Methylfucose	2.08	1.71	11.2
Fucose	2.20	1.90	16.2
2,4,6-Tri-*O*-methylgalactose	2.20	1.96	10.6
2,3,6-Tri-*O*-methylgalactose	2.45	2.10	5.0
Di-*O*-methylgalactose	3.60	2.70	small

[a] The retention times are given relative to 2,3,4,6-tetra-*O*-methyl-D-glucitol 1,5-diacetate. [b] Hidden in the neighbouring peaks.

polysaccharide molecule. Had it been present in the interior part, smaller fragments containing considerable amounts of carbohydrate would have passed through the dialysis bag during dialysis; this did not take place.

Methylation studies. Both the original polysaccharide and the product after the second periodate oxidation were methylated, [9] hydrolysed and converted into the partially methylated alditol acetates.[7] The mixtures were analysed on GLC (columns 1 and 2), and the identification of the products, confirmed by mass-spectrometry, is presented in Tables 3 and 4. The retention times obtained correspond quite well with those obtained by Lönngren and Pilotti.[10] For the derivatives of the original polysaccharide, the areas under the peaks are given in Table 3, allowing a semiquantitative estimation of the relative amounts of the methylated derivatives.

The methylation results show that all three sugars are present as end groups, rhamnose being responsible for the main part, followed by galactose and fucose as the minor part.

The rhamnose residues which are not present as end groups are mainly 1,2-linked as shown by the large proportion of 3,4-di-*O*-methylrhamnose and were accordingly vulnerable to periodate attack, in good agreement with the periodate results described above. A small proportion of rhamnose appears to be 1,3-linked and the possibility of this sugar occurring as branch points is indicated by the presence of small amounts of 2-*O*-methylrhamnose.

The galactose residues in the chain are mainly 1,3-linked, giving rise to 2,4,6-tri-*O*-methylgalactose. A smaller amount is 1,4-linked, and these residues, together with the end groups were oxidised in the first periodate treatment. The possibility of galactose forming a few branch points in the molecule is indicated by traces of di-*O*-methylgalactose.

Fucose, however, forms the main part (> 90 %) of the branch points in this poly-

Table 4. GLC analysis of the methylated alditol acetates from the product obtained after 2nd. periodate oxidation.

Identity	Retention times[a]	
2,3,4,-Tri-*O*-methylrhamnose	0.43	S[b]
2,3,4-Tri-*O*-methylfucose	0.6	S
Unknown	0.84	S
3,4-Di-*O*-methylrhamnose	0.90	S
2,4-Di-*O*-methylfucose	1.02	L
2,3,4,5-Tetra-*O*-methylgalactose	1.18	S
2-*O*-Methylrhamnose	1.35	S
2-*O*-Methylfucose	1.42	M
4-*O*-Methylfucose	1.73	L
2,4,6-Tri-*O*-methylgalactose	1.89	L

[a] See Table 3. [b] Relative size of peaks: L = large, M = medium, S = small.

saccharides, being to a large extent recovered as free fucose and 2- and 4-*O*-methylfucose. A small proportion of the fucose was recovered as 2,4-di-*O*-methylfucose, showing the presence of 1,3-linked fucose residues in the molecule.

The methylation of the polyalcohol obtained after the reduction of the product remaining after the second periodate oxidation gave 2,4,6-tri-*O*-methylgalactose and 2,4-di-*O*-methylfucose as the main products, indicating that the Smith degradation had removed some of the side chains attached to the fucose residues. The polymer was, however, still branched, indicated by the presence of mono-*O*-methylfucose.

The polysaccharide was not desulfated before methylation. No systematic investigation of the Hakomori methylation procedure on sulfated polysaccharides has been carried out, but complete methylation by this procedure of highly sulfated polysaccharides has proved impossible,[18] indicating that the methylation procedure does not lead to desulfation. The presence of free fucose and some of the methylderivatives indicating three substituents may, thus, originate from sulfated sugar residues and not from branch points. This is indicated by the results given in Table 3; the area under the peaks corresponding to free fucose and to mono-*O*-methylfucose and rhamnose and di-*O*-methylgalactose is considerably larger than the area under the peaks corresponding to end groups. The possibility of incomplete methylation should, however, also be considered. The position of sulfate is not known, but a band at 850 cm^{-1} in the IR spectrum indicates that it is present on an axial, secondary hydroxyl group.[11,12] This can be C-4 on D-galactose in $^4C_1$ conformation, C-2 on L-rhamnose in $^1C_4$, or C-4 on L-fucose in $^1C_4$ conformation. A small band at 820 cm^{-1} was also present. This indicates sulfate on a primary hydroxyl group, which, in this polysaccharide, would be C-6 on D-galactose.

The results described above show the extracellular polysaccharide produced by the diatom *C. affinis* to be a highly branched polymer of a complex structure. The rhamnose residues occur mainly in the outer part of the molecule, and the major part of the end groups of the molecule are rhamnose, although fucose and galactose also occur as end groups. The branch points in the molecule are fucose residues.

EXPERIMENTAL

Hydrolysis. The sample was dissolved in 90 % formic acid [13] and heated in a sealed tube for 6 h at 100 °C. After dilution with 5 volumes of water, the mixture was heated for another 2 h. The formic acid was removed by several evaporations to dryness after addition of methanol.

Preparation of alditol acetates (7). After hydrolysis the sugars were reduced to the corresponding alditols using $NaBH_4$. A mixture of acetic anhydride and pyridine (1 + 1) was added, followed by heating at 100 °C in sealed tubes for 1 h. 2 ml water was added and heating continued in a boiling waterbath for another 15 min. The derived alditol acetates were extracted with chloroform and analysed by GLC.

Gas liquid chromatography (GLC) of the partially methylated alditol acetates was performed on 0.3 × 200 cm stainless steel column in a Varian 1400 gas chromatograph, using 3 % ECNSS-M on Varaport-30 (column 1) at 165 °C and 0.3 × 400 cm glass column using 3.5 % OV-225 on Varaport-30 (column 2) at 185 °C.

Alditol acetates of the sugars were analysed as described by Myklestad *et al.* [4]

Carbohydrate contents were measured by the phenol-sulfuric acid method [5] using a mixture of rhamnose, fucose, and galactose in the proportions 35:39:26 as standard.

The proportions of sugars present in the hydrolysates were determined by GLC.

Sulfate content was estimated by potentiometric titration and after Antonopoulos method. [6]

IR-spectra were recorded in a Perkin-Elmer 257-spectrometer.

Dialysis was always carried out against distilled water.

The *polysaccharide* was prepared as described elsewhere.[2,4]

Periodate oxidation was performed in acetate buffer at pH 4.0, using excess periodate. The reaction was followed by withdrawing aliquots of 50 µl, which were diluted to 50 ml and OD read at 223 nm.[6] The reaction was complete after 4 h and excess periodate destroyed by adding ethylene glycol. The polyaldehyde was reduced to the corresponding polyalcohol with $NaBH_4$. Excess $NaBH_4$ was destroyed and the solution neutralized with acetic acid. The product was dialysed and freeze-dried.

Weak acid hydrolysis (1 N H_2SO_4 for 4 h at room temperature) was carried out in order to cleave acetal-linkages. The hydrolysate was neutralised with Na_2CO_3 and the final products freeze-dried after dialysis.

This polysaccharide was subjected to another periodate oxidation like the one mentioned above. The reaction was followed by withdrawing samples of 100 µl which were diluted to 25 ml. Carbohydrate contents and sugar-ratios were measured after all the above-mentioned steps.

Methylation.[14] The polysaccharide (10 mg) was freeze-dried, dried *in vacuo* over P_2O_5 overnight and dissolved in 1 ml dimethyl sulfoxide (DMSO) in a McCarthey-bottle.[9] Methylsulfinyl sodium in DMSO (1.5 ml, 2 M)[15] was added and the reaction mixture was stirred vigorously for 4 h. Methyl iodide (0.5 ml) was added while cooling, and stirring continued for another 2 h. The reaction mixture was then poured into water and dialysed against distilled water several times. The methylated product was hydrolysed, the partially methylated sugars converted into the corresponding partially methylated alditol acetates, and analysed by GLC on columns 1 and 2. The identity of the peaks were confirmed by GLC (column 2) combined with mass-spectrometry,[16] carried out on a combined GLC-MS instrument, type Varian CH7. Instrumental details are published elsewhere.[17]

Methylation of the product obtained after *the second periodate oxidation.* The polymer was methylated, converted to the partially methylated alditol acetates as described above, and analysed by GLC on column 2.

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