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CHEMISTRY OF THE POLYSACCHARIDES OF THE DIATOM *COSCINODISCUS NOBILIS*

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Abstract—The extracellular polysaccharide of *Coscinodiscus nobilis*, a member of the Coscinodiscaceae, contains a highly branched heteropolysaccharide(s) containing fucose, rhamnose, mannose, D-glucose, xylose, D-glucuronic acid, galactose (trace) and half ester sulphate. The positions of linkages between the monosaccharides have been established and evidence for the linkages between D-glucuronic acid and monosaccharides was obtained. The extracellular polysaccharide contained also a chrysolaminaran, but this may have been derived from dead cells. Fucose and mannose occur also in a separate polymer. The diatom contained polysaccharide material consisting of glucose, mannose, fucose and uronic acid residues.

INTRODUCTION

It is known that several diatoms produce extracellular polysaccharides, for example *Nitzschia frustulum* [1], *Thalassiosira gravida* (Cleve), *T. fluviatilis*, *Skeletonema costatum* [2] and several members of the *Chaetoceros* genus [3]. The structures of the sulphated polysaccharides of the latter have been investigated. Those of *C. affinis* [4] and *C. curvisetus* [5] seem to have similar structures and contain rhamnose as end groups, (1→2)-linked rhamnose, (1→2)- and (1→4)-linked galactose, (1→3)-linked fucose and branching fucose residues. In addition, the polysaccharide of *C. curvisetus* possessed the unusual feature of containing both furanoid and pyranoid residues of fucose.

The diatom *Coscinodiscus nobilis* is normally absent from European waters, but appeared in the early part of 1977 in large numbers in the western English Channel [6]. Its extracellular mucilage, most prevalent on the sea bed, made trawling extremely difficult. In consequence, we have collaborated with the Marine Biological Association of the U.K. to culture this diatom, and we have investigated the diatom's extracellular polysaccharides, the polysaccharides present in the diatom, as well as extracts from the sea bed mud. Our investigations remain, however, fragmentary as the diatom vanished from the English Channel and neither the Laboratory of the Marine Biological Association nor we were able to maintain a culture of the diatom. In consequence only small quantities of materials were available. Nevertheless, we report our findings for two reasons. Firstly, comparatively little is known about polysaccharides of the ecologically important diatoms, and our results may be of intrinsic interest. Secondly, the appearance of *C. nobilis* in the English Channel caused a serious handicap to fishing.

RESULTS AND DISCUSSION

The hydrolysates of the crude mucilage (**A**) and the extract (**B**) from the sea bed mud were shown, by PC and GC–MS, to contain fucose, rhamnose, mannose, glucose, galactose (trace), and paper electrophoresis [7] revealed glucuronic acid. Reaction with D-glucose oxidase showed that the glucose was the D-isomer. The hydrolysates also contained amino acids. It is therefore likely that the extracellular mucilage produced by *C. nobilis* was the cause of the slimy nature of the sea bed mud which was damaging fishing nets.

The amount of extracellular polysaccharide material (**C**) produced by cultured *C. nobilis* (*ca* 30 mg/dm^3) is comparable with that of other cultured diatoms. This material, which was sulphated contained the monosaccharides fucose, rhamnose, mannose, D-glucose, xylose, D-glucuronic acid and galactose in the approximate mol. -percentages of 34, 15, 19, 16, 6, 9, trace, respectively. These monosaccharides, except xylose, were also found [8] in the hydrolysate of whole cells of *Coscinodiscus* sp., although in different proportions. Major differences between the extracellular polysaccharide of *C. nobilis* and those of other diatoms so far investigated are the absence of (a) fucose from the *N. frustulum* polysaccharide [1] and (b) mannose and glucuronic acid from the polysaccharides of *Chaetoceros* sp. [3].

Fractionation of the polysaccharide material (**C**) showed this to be composed of at least three different polymers, namely a glucan, a polymer containing all the above monosaccharides except glucose, and a polymer containing mannose and fucose. Although lack of material prevented larger scale investigations of the glucan, the results of the methylation analysis of the unfractionated polysaccharide material (**C**) showed

that the major linkages in the glucan are of the (1→3) type and that it also contains a small proportion of branching glucose units linked through C-1, C-3 as well as C-6. The characterization of D-glucose as the major product of periodate oxidation of the polysaccharide material (**C**), followed by reduction and hydrolysis, together with the negative optical rotatory power of the glucan ($[\alpha]_D^{20}$ −7.5° (*c* 0.2; H_2O)), indicate that it is a chrysolaminaran. The presence of a fairly high proportion of 2,3,4,6-tetra-*O*-methylglucose in the hydrolysate of the methylated polysaccharide material (**C**) indicates that this laminaran is a relatively small molecule in keeping with the chrysolaminaran found in other diatoms. It could not be ascertained whether the chrysolaminaran is an extracellular polysaccharide or whether it was derived from cells which had died and disrupted during the 10 weeks of incubation.

Two applications of the methylation analysis procedure to the polysaccharide material (**C**) gave a complex mixture of *O*-acetyl-*O*-methylalditols. Their GLC retention times, together with the *O*-methylmonosaccharides from which they are derived, are shown in Table 1. The results show that all neutral monosaccharides occur in the polymer as non-reducing end groups (it is appreciated that some of these could be the result of degradation caused by the methylation reagents), and that the major chain-units are (1→3)-linked fucose, (1→2)-linked rhamnose, (1→6)-linked mannose, and (1→3)-linked D-glucose (see above). Fucose and rhamnose occur as branching units or may be sulphated. The major portion of xylose occurs as non-reducing end groups.

Table 1. Products of methylation analysis of polysaccharide material (**C**)

Acetylmethyl alditol (*T*)*	Parent *O*-methylmonosaccharide
0.47 (m)	2,3,4-tri-*O*-methylrhamnose†
0.55 (m)	2,3,4-tri-*O*-methylxylose†
0.61 (vl)	2,3,4-tri-*O*-methylfucose†
0.88 (m)	3,4-di-*O*-methylrhamnose†
1.02 (vl)	2,4-di-*O*-methylfucose
1.16 (l)	2,3,4,6-tetra-*O*-methylglucose†
	2,3,4,6-tetra-*O*-methylmannose
	2,3-di-*O*-methylxylose
1.27 (m)	2-*O*-methylrhamnose†
1.41 (m)	2-*O*-methylfucose†
1.59 (s)	4-*O*-methylrhamnose†
1.71 (l)	3-*O*-methylfucose/rhamnose†
1.81 (l)	2,4,6-tri-*O*-methylglucose†
1.98 (s)	2,4,6-tri-*O*-methylmannose†
2.16 (s)	2,3,6-tri-*O*-methylmannose†
2.25 (l)	2,3,4-tri-*O*-methylmannose†
2.55 (s)	2,3,6-tri-*O*-methylglucose†
3.88 (s)	3,6-di-*O*-methylglucose/mannose
4.3 (s)	3,4-di-*O*-methylmannose†
	2,4-di-*O*-methylglucose†
4.79 (s)	2,4-di-*O*-methylmannose†

* Retention time relative to 1,6-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; Peak size: vl = very large, l = large, m = medium, s = small.

† Structure of acetylmethylalditol confirmed by MS.

No methylated uronic acids were detected in the product of the methylation analysis procedure of the material (**C**), but it contained components with R_{TMG} 0–0.24. Further fractionation followed by esterification, reduction and hydrolysis showed that these were indeed unhydrolysed oligouronic acids. The characterization of glucose (derived from glucuronic acid), fucose/rhamnose and xylose indicates that these oligouronic acids were either highly branched or that incomplete methylation had occurred. The results nevertheless show that in the polymer some of the fucose, rhamnose and xylose are in close proximity to the D-glucuronic acid.

Sequential extraction of the diatom with water and alkali gave small quantities of polysaccharides. The aqueous extract gave on hydrolysis glucose, mannose and fucose. It is likely that this glucose derived from chrysolaminaran. The alkali extract gave on hydrolysis mannose and uronic acid. The presence of a sulphated glucuronomannan in a similar extract from *Phaedactylum tricornutum* [9] indicates a possible similarity between these two genera of diatoms.

EXPERIMENTAL

Materials. (a) A sample of mucilage was supplied by Dr. G. T. Boalch (Marine Biological Association of the U.K., Plymouth), which, after removal of inorganic materials with Amberlite IR-120 (H^+) and Amberlite IR-45B (OH^-), was freeze-dried to a solid (**A**; 9.6 mg, carbohydrate content 20%). (b) A sample of wet sea clay (*ca* 200 g; supplied by Dr. G. T. Boalch) was extracted twice with 80% EtOH (600 cm^3) at room temp. for 18 hr. Residue was dried at room temp. and powdered, and all foreign particles, such as Crustaceae, small particles of seaweed and metal, were removed. Residual powder was extracted with deionized H_2O (once with 2 dm^3 for 18 hr and again with 1 dm^3 for 6 hr). Combined extracts were freeeze-dried to a solid (906 mg; found: carbohydrate, 12.4; protein, 7.9%). The solid was redissolved in H_2O and dialysed exhaustively against deionized H_2O, the dialysate containing only a negligible amount of carbohydrate. The dialysed material was freeze-dried (**B**, 250 mg; found: carbohydrate, 62.4, protein 11.3%). (c) The diatom was cultured in 6×200 cm^3 Erd–Schreiber medium [10]. After 10 weeks the carbohydrate content of the culture solution was constant. Combined media were centrifuged at 740 g for 1 hr. The centrifugate was washed (H_2O) and dried at room temp. to give the cultured diatom (**D**) (30 mg). Supernatant was dialysed against distilled H_2O for 3 days and freeze-dried to a solid (76 mg). It was dissolved in H_2O (5 cm^3) and EtOH (25 cm^3) added slowly with stirring. The derived ppt. was removed, redissolved in H_2O and freeze-dried to a colourless solid (**C**, 50 mg; $[\alpha]_D^{20}$ −4.6° (*c* 0.54)); found; carbohydrate, 63; uronic acid, 9.3; sulphate, 16.7%).

General methods. (a) Details of analytical methods are given in ref. [11]. (b) *GLC.* A Pye 104 gas chromatograph was used, containing a glass column (3 m×5 mm) packed with 3% of OV225 on Chromosorb Q (100–120 mesh) and with N_2 as carrier gas and a flame ionization detector. (c) *GC–MS.* The gas chromatograph (Pye 104 as above) was interfaced with a VG Micromass 12 F mass spectrometer. For EI–MS the mass spectrometer was operated at 70 eV, with a target current of 20 μA and an ion-source temp. of 200°. For

CI–MS the reagent gas was isobutane at a pressure of *ca* 0.4 torr, and the ion-source was operated at *ca* 150°, 50 eV and an emission current of 1000 μA.

Acid hydrolysis of polysaccharide material (**C**), *characterization and determination of monosaccharide products.* A portion (*ca* 10 mg) of the polysaccharide was hydrolysed. Hydrolysate was fractionated, using Whatman No. 3MM paper and solvent C [11], into neutral and acidic material. A measured portion of the neutral material was converted into *O*-acetylalditols (by reduction with $NaBH_4$, followed by acetylation with Ac_2O–Py). These were identified by GLC and the relative molecular porportions of fucose/rhamnose, mannose, glucose, xylose and galactose (3:1.15:1:0.3, trace, respectively) calculated from the peak areas. Another portion of the neutral material was fractionated on Whatman No. 1 paper using solvent B [11]. The separate fractions containing fucose and rhamnose were filtered through millipore (pore size 0.45 μm) each monosaccharide was determined [11] and the molecular ratio of fucose: rhamnose calculated to be, respectively, 2.3:1. A measured portion of the acidic material was esterified with diazomethane, reduced with $NaBH_4$ and hydrolysed. PC revealed a single product with a migration rate identical with that of glucose. Its reaction with D-glucose oxidase showed it to be D-glucose. The amount of D-glucuronic acid relative to that of the neutral materials was determined, using the phenol–H_2SO_4 method [11], by comparison of a measured portion of the acid material with standard solutions of D-glucuronic acid, and that of the neutral material with standard solutions containing fucose, mannose, glucose and xylose in the molecular proportions of 3:1.15:1:0.3. The ratio of D-glucuronic acid to neutral monosaccharides was calculated to be 1:10, in close agreement with the uronic acid content (9.3%) of the polysaccharide material (**C**) determined by the carbazole method.

Fractionation of polysaccharide material (**C**) *on DE-52 cellulose.* The polysaccharide (5.6 mg carbohydrate) dissolved in water (1 cm^3) was applied to a column (18 × 1.5 cm) of DE-52 cellulose (pre-swollen microgranular) which had been equilibrated with 0.5 M KCl. The column was eluted with H_2O (*ca* 150 cm^3, fraction 1), until the eluant was carbohydrate-free and then with 0.3 M KCl (*ca* 150 cm^3, fraction 2), M KCl (*ca* 150 cm^3, fraction 3) and M NaOH (*ca* 150 cm^3, fraction 4). The polysaccharide in each fraction was hydrolysed and the monosaccharides of each hydrolysate were characterized (by PC) and, after conversion into corresponding *O*-acetylalditols, by GC–MS) as follows. Fraction 1: glucose and trace of fucose; fraction 2: all monosaccharides of the polysaccharide (**C**) except glucose; fraction 3: nil; fraction 4: mannose and fucose.

Methylation of polysaccharide material (**C**). A portion of the polysaccharide (5.6 mg carbohydrate) was twice methylated by the Hakomori method. The methylated material was hydrolysed and examined by PC using solvents A, B and C. A portion of the hydrolysate was reduced (with $NaBH_4$) and converted into *O*-acetyl-*O*-methylalditols (with Ac_2O–Py), which were analysed by GC–MS. The results are shown in Table 1. The remainder of the above hydrolysate was fractionated on Whatman No. 3 MM paper using solvent A and fractions with R_{TMG} 0.24 (fraction 1), 0.11 (fraction 2), 0.04 (fraction 3), and 0 (fraction 4) obtained. Each fraction was separately esterified (with CH_2N_2), reduced (with $NaBH_4$) and hydrolysed (with 0.5 M H_2SO_4). These hydrolysates were neutralized with *N*-methyldioctylamine (5% in $CHCl_3$). Portions of these hydrolysates were examined by PC and, after conversion into *O*-acetyl-*O*-methylalditols (see above), by GC–MS. The results showed that the products of reduction and hydrolysis were fucose/rhamnose (fractions 1, 2, 3 and 4), xylose (fractions 2, 3 and 4), 2,3-di-*O*-methylfucose (fraction 2), 2,3-di-*O*-methylglucose (fraction 4), 2-*O*-methylglucose (fractions 2, 3 and 4) and glucose (fractions 1, 2, 3 and 4).

Periodate oxidation of polysaccharide (**C**). The polysaccharide (2.78 mg carbohydrate) was treated in the dark with 5 mM $NaIO_4$ (5 cm^3). Examination [12] of aliquots showed that after 48 hr the oxidation was complete. Excess periodate was reduced by addition of ethylene glycol (0.2 cm^3). $NaBH_4$ was added, the whole left at room temp. for 3 hr and then at 2° for 18 hr. The soln was dialysed until free from inorganic materials and then freeze-dried. The product was hydrolysed, and PC and reaction with D-glucose oxidase revealed D-glucose as the major component in the hydrolysate.

Carbohydrates in the diatom (**D**). The cultured diatoms (30 mg) were sequentially extracted with cold H_2O (20 cm^3) for 48 hr, H_2O (20 cm^3) at 80° for 6 hr, and after chlorite treatment at 70° [13], with M NaOH. The final residue was devoid of carbohydrates. Each extract was freeze-dried separately and analysed for carbohydrates.

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