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New structural insights into the cell-wall polysaccharide of the diatom *Phaeodactylum tricornutum*

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ABSTRACT

The cell wall of diatoms is composed of silica and organic matter, including protein, long-chain polyamines and polysaccharides. The main polysaccharide, a sulphated glucuronomannan, has been observed in many diatoms suggesting that it plays an important role in cell-wall biogenesis. We extracted and purified the polysaccharide, prepared oligosaccharide series and analysed the structure of poly- and oligosaccharides using chromatography and NMR spectroscopy methods. Our results show that the backbone of the polysaccharide is predominantly a linear poly-α-(1 → 3) mannan decorated with sulphate ester groups and β-D-glucuronic residues.

1. Introduction

The diversity of carbohydrate residues and the number of their possible linkages explain the wide structural diversity of polysaccharides. The carbohydrate polymer backbone is often decorated with organic (e.g. methyl, pyruvate, acetate) or inorganic derivatives (e.g. sulphate), further increasing the complexity of these macromolecules. In the oceans, polysaccharides represent the most abundant biomass produced by photosynthetic organisms, including uni- and multicellular species: micro- and macroalgae, respectively. Structural analyses of marine polysaccharides have primarily focused on the cell-wall polysaccharides of macroalgae due to their economic importance, e.g. as sources of agar and carrageenan (red algae), alginate and fucoidan (brown algae) and ulvan (green algae). Several papers have reviewed the structural and complexity of macroalgal polysaccharides and their applications [1–4].

Microalgae play a significant role in the ocean biogeochemical cycle; in particular, in terms of the fixation of global carbon, they account for about 20% of total photosynthesis [5]. These organisms have attracted more and more attention and harbour economic value as sources of novel molecules, as producers of lipids for bioenergy and as cell factories [6–9]. Despite the recognized importance of these organisms, the structure of microalgal polysaccharides has been virtually unexplored in contrast to those of macroalgae. Composition analyses of microalgal polysaccharides located in the cell wall, secreted or used for energy storage reflect the very broad diversity of polysaccharide structures [10,11]. However, only very few structures of microalgal polysaccharides have been resolved due to difficulties encountered in extracting pure samples and the complexity of their chemical structure. For example, advanced structural data is available for only three microalgal polysaccharides: on the extracellular polysaccharide of the red microalga *Porphyridium* sp. [12], the cell-wall polysaccharide of *Chlorella* sp. [13–15] or the theca polysaccharides of *Tetraselmis* sp. [16–18].

Among microalgae, diatoms (Bacillariophyta) are characterised by a cell wall made of amorphous silica (SiO_2) and organic molecules including polysaccharides, proteins (e.g. frustulins, pleuralins, silaffins) and long-chain polyamines [19–21]. The mechanism behind the elaborate architecture of the diatom cell wall is not understood in vivo; nevertheless, it has been demonstrated that poly-condensation of silicic acid can be controlled in vitro in presence of phosphorylated silaffins and long-chain polyamines and leads to silica particles, thereby mimicking the process observed in native cell wall [22–24].

In contrast with other diatoms, the cell wall of *Phaeodactylum tricornutum* is poor in silica and mainly composed of organic molecules, notably, a sulphated glucuronomannan [25–27]. The polysaccharide backbone is made of a mannan chain decorated with sulphate ester group. The configuration of all the glycosidic linkages were not elucidated but optical rotation measurement suggested that the polysaccharide contained mainly α-bonded residues. Based on structure analyses of fragments obtained by mild acid hydrolysis, it was proposed that branching are made of mannose and glucuronic acid. Despite the partial structure of *P. tricornutum* polysaccharide, this sulphated

Abbreviations: GC, gas chromatography; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum correlation

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glucuronomannan has been observed in many other diatom species, suggesting that it has an important and conserved biological function among diatoms [11,19,28,29]. The polysaccharide is thought to play a role in shaping the architecture of the frustule, but the mechanism remains obscure [21]. In addition, *P. tricornutum* is considered to be a model organism for the study of diatom biology and its genome has been sequenced [30,31]. Therefore, *P. tricornutum* is also a model of choice to investigate cell biogenesis and the assembly of organic and inorganic components. In this context, we revisited the pioneering work of Percival and McDowell (1967) [27] using modern analytical methods and we report, here, the most advance description of the main cell wall polysaccharides *P. tricornutum* as a diatom model organism.

2. Experimental

2.1. Production, isolation and purification of the Phaeodactylum tricornutum cell-wall polysaccharide

Lyophilised cells of *P. tricornutum* in fusiform morphotype were provided by Fitoplancton Marino (El Puerto de Santa Marine, Spain). The strain belongs to the Microalgae Culture collection of Fitoplancton Marino, SL (CCFM) under the accession number CCFM 06. The cell-wall polysaccharide was extracted as described in Ford and Percival [25]. Pigments and lipids were Soxhlet-extracted with ethanol (pure cellulose extraction thimbles, grade 603, 1.5 mm thickness; extraction period of 4 days). After drying, the storage polysaccharide (β-glucans) was dissolved in hot water (80 °C, 2 h) and the cell pellets were recovered after centrifugation (6000 rpm, 15 min). The hot-water extraction was repeated twice. The remaining insoluble material was then bleached at 80 °C for 3 h using a solution made with 200 mL of a chlorite solution (NaClO4 monohydrate; 1.7% in water) mixed with 200 mL of acetate buffer (27 g NaOH mixed with 75 mL glacial acetic acid completed to 1 L with water) and completed to 1 L with deionised water. The sample was then transferred by centrifugation into 1% NaOH and maintained in the alkaline solution for 1 h under agitation. The bleaching and alkaline treatment was repeated three times. The cell wall polysaccharide was finally dissolved at 100 °C for 45 min in 4% NaOH containing 20 mM $NaBH_4$. After dialysis against distilled water (cut-off 1 kDa), the recovered cell-wall polysaccharide was freeze-dried.

2.2. Molecular characteristics

2.2.1. Molecular weight

The molecular weight of the *P. tricornutum* cell-wall polysaccharide was determined by high-performance size-exclusion chromatography (HPSEC) using a three-angle light scattering detector (MiniDAWN™ TREOS), coupled with a refractive index detector OptiLab Rex Wyatt. Elution was performed at 0.5 mL/min on two Shodex™ OHpak™ SB-806M columns mounted in series (exclusion limit 2.10^7 g/mol) with 0.1 M $NaNO_3$ as the eluent. The molecular mass was calculated using a dn/dc value of 0.142.

2.2.2. Sulphate content

Elemental analysis was performed by the CNRS Microanalysis Facility (SCA, Villeurbanne, France). Sulphate content (sodium salt) was calculated from the sulphur analysis based on the following equation: sulphate ester group = 3.2 × S% (w/w) and then divided by the amount of glycosyl.

2.3. Monosaccharide analysis

The molar ratio of monosaccharides was determined according to Kamerling et al. [32] modified by Montreuil et al. [33]. The polysaccharide was hydrolysed by 3 M MeOH/HCl at 100 °C for 4 h, followed by *N*-acetylation with pure anhydride Ac_2O overnight at room temperature. The methyl glycosides were converted to their corresponding trimethylsilyl derivatives. Separation and quantification of the per-*O*-trimethylsilyl methyl glycosides were performed by gas chromatography (GC) using an Agilent system equipped with a HP-5MS capillary column (0.25 mm × 30 m). The trimethylsilyl derivatives were analysed using the following temperature program: 120 °C for 1 min, 120 °C → 180 °C at 3 °C/min, 180 °C → 200 °C at 2 °C/min, 200 °C for 5 min. For the tetraol analysis, the temperature program was 90 °C for 1 min, 90 °C → 200 °C at 2 °C/min, 200 °C for 5 min.

The absolute configuration of the sugar residues was established from native and carboxyl-reduced polysaccharides, based on the GC analysis of the derived trimethylsilylated (*S*)-2-butylglycosides and comparison of their retention times with reference compounds [34]. The derived butylglycosides were separated using the following temperature program: 120 °C for 1 min, 120 °C → 135 °C at 3 °C/min, 135 °C → 200 °C at 1 °C/min.

2.4. Methylation analysis

Glycosyl-linkage positions were determined as described [35]. Hydroxyl groups were methylated using the lithium dimethylsulfonyl as the anion and methyl iodide in Me_2SO [36,37]. After two methylation steps, the permethylated products were hydrolysed in 2 M trifluoroacetic acid (TFA) for 2 h at 120 °C, then reduced with $NaBD_4$ in a NH_4OH solution for 30 min at 80 °C, and finally acetylated with 1:1 Ac_2O:pyridine (v:v) for 30 min at 120 °C. Uronic acids were reduced before methylation using carbodiimide method [38] or after methylation using Linneborg method [39], with $LiBD_4$ prepared in situ with $NaBH_4$ and LiCl [40]. GC-mass spectrometry (MS) was performed on an Agilent instrument fitted with a Supelco SP2380 capillary column (0.53 mm × 60 m). The temperature program was 150 °C for 2 min, 150 °C → 240 °C at 3 °C/min, 240 °C for 5 min. Ionisation was carried out in electron impact mode (EI, 70 eV). All analyses were conducted in triplicate.

2.5. Mild acid hydrolysis

Oligosaccharides were generated by mild acid hydrolysis of the polysaccharide (1 g/L) in 0.2 N TFA at 100 °C for 4 h. The product was neutralised with barium carbonate and filtrated on 0.22 μm membrane (Nylon, Jasco syringe filters). A first fractionation was achieved with a HiLoad™ 26/600 Superdex™ 30 column. Then the fractions were re-injected and separated with three HiLoad™ 26/600 Superdex™ 30 columns mounted in series (1.2 mL/min, 0.1 M NH_4OAc buffer). After checking the purity of the fractions using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex) on PA100 columns (Dionex), the fractions were pooled.

2.6. Smith degradation

Smith degradation of the polysaccharide was done according to [41]. Briefly, 100 mg of polysaccharide were oxidised with 100 mL of 50 mM $NaIO_4$ at 4 °C. After 5 days in the dark, the excess of oxidant was destroyed by the addition of ethylene glycol. The oxidised polysaccharide solution was reduced by 20 mL of $NaBH_4$ (15 mg/mL in 9:1 EtOH:H_2O (v:v)), dialysed against distilled water for 48 h (6000–8000 Mw cut-off) and freeze-dried. The reduced product was hydrolysed with acetic acid (1.5%) for 2 h at 100 °C, neutralised by co-evaporation with methanol and freeze-dried again. The samples were fractionated by gel-permeation chromatography on a Toyopearl HW-40 Gel column (1.6 × 100 cm) at a flow rate of 0.4 mL/min with 0.1 M NH_4OAc as buffer. The full process was repeated five times to accumulate enough product for subsequent experiments.

2.7. NMR spectroscopy

Samples were deuterium-exchanged three times by freeze-drying in