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STRUCTURE OF A FOOD-RESERVE β-D-GLUCAN PRODUCED BY THE HAPTOPHYTE ALGA *Emiliania huxleyi* (LOHMANN) HAY AND MOHLER*

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ABSTRACT

The title alga grown in batch culture under nitrogen-limitation conditions contained 16% of a water-soluble β-D-glucan having $[\alpha]_D^{20}$ −10° and an average d.p. of 106. Methylation analysis, Smith degradation, and ^{13}C-n.m.r. spectroscopy indicated that the glucan consists of a mainly (1→6)-linked backbone, substituted at positions 3 with (1→6)-linked side-chains having an average length of two D-glucose residues.

INTRODUCTION

Most algae store energy as either α- or β-D-glucans, and these are regarded as important taxonomic characteristics. The latter group are usually assumed to be laminarans, but there are whole Phyla for which proof of this is lacking[1,2]. Although the function of many of the polysaccharides of algal cells is not clear, the glucans are generally considered as storage polysaccharides[1]. We now report on the isolation and the primary structure of a reserve β-D-glucan from *Emiliania huxleyi*, which is a coccolithophorid, belonging to the class Haptophyceae, and which contains water-soluble polysaccharides[3].

RESULTS AND DISCUSSION

The alga was grown in batch culture, and the acid-extractable, water-soluble glucan was accumulated in the stationary growth phase (with nitrate as the limiting nutrient), indicating a storage function[4]. Extraction of the freeze-dried alga with aqueous acid gave a polysaccharide in a yield of 16.2% (see Experimental), which had $[\alpha]_D^{20}$ −10° (*c* 0.8, water). G.l.c. of the alditol acetates of the products of acid

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hydrolysis indicated the presence of >99% of glucose and a trace of mannose. The glucose in the hydrolysate could be quantified by the D-glucose oxidase method[5], indicating its D configuration. The average d.p. of the glucan, determined by the measurement of reducing power, was 106.

The glucan was subjected to methylation analysis, and the resulting alditol acetates were analysed by g.l.c.–m.s.[6,7] (Table I). It was also subjected to Smith degradation (periodate oxidation, borohydride reduction, and mild acid hydrolysis). The product was eluted from a column of Biogel P2 in the void volume, indicating a molecular weight of >2,000 for the periodate-resistant polymer. These results indicated that the glucose residues linked only at positions 1 and 6 were located in the side chains.

The ^1H-decoupled ^{13}C-n.m.r. spectrum of a solution of the glucan in D_2O at 80° showed major signals at δ 104, 87–86, 77–76, 74, 71–70, 70–69, and 62 (Fig. 1). These data correspond to a (1→3),(1→6)-linked β-D-glucopyranan structure, since all the C-1 signals had chemical shifts characteristic of the β configuration[8], and the chemical shifts of the other signals were different from those of β-(1→2)-[9] and β-(1→4)-linked[10] structures. The signals of the C-3 carbons involved in glycosidic bonds were found at δ 87.2 and 86.0 and were broad. It is assumed that the downfield signal was due to C-3 in a (1→3),(1→6)-linked D-glucose residue and the other signal to C-3 in a (1→3)-linked D-glucose residue.

The assignment of the broad resonance at δ 69.6 to C-6 of a (1→6)-linked D-glucose residue[11] was confirmed by a DEPT n.m.r. pulse sequence, which identified this signal as due to a CH_2 group[12]. The signals at δ 61.9 and 62.0 were due to CH_2OH groups. These data are summarised in Table II. Because of the complexity of the spectrum, no attempt was made to identify unequivocally all the carbon resonances. The peak areas allowed the relative amounts of the different carbon atoms to be estimated (with the n.O.e. suppressed). The fraction of D-glucose residues linked at position 6 was estimated to be 64% [area of C-6(linked/total area of C-6], in agreement with the result (58%) of g.l.c.–m.s., and the fraction linked at position 3 was estimated to be 35% (area of C-3/area of C-1), which also accorded with the g.l.c.–m.s. result (38%).

It was not possible to determine from the n.m.r. and the g.l.c.–m.s. data whether the main chain was (1→3)-linked with branch points at positions 6, or

TABLE I

G.L.C. DATA FOR THE METHYLATED ALDITOL ACETATES

Alditol acetate derived from	*Primary fragments* (m/z)	*Peak area (%)*
2,3,4,6-Tetra-*O*-methylglucose	45–117–161–205	34
2,4,6-Tri-*O*-methylglucose	45–117–161–233	7.3
2,3,4-Tri-*O*-methylglucose	117–161–189–233	28
2,4-Di-*O*-methylglucose	117–189–233–305	31

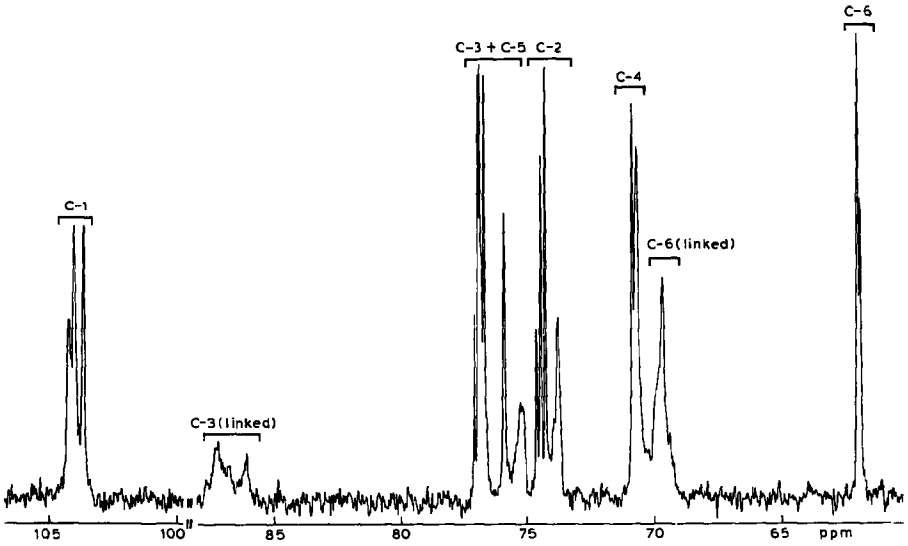


Fig. 1. ^{13}C-N.m.r. spectrum (100 MHz) of the glucan isolated from *E. huxleyi*.

(1→6)-linked with branch points at positions 3. However, n.m.r. spectroscopy showed that the non-dialysable Smith-degraded glucan, which must contain the main chain of the native glucan, was essentially (1→6)-linked (Fig. 2). A small amount of unsubstituted C-6 was indicated by the signal at δ 62, together with glycosidically linked C-3 at δ 85. These signals arose from the otherwise unsubstituted 3-linked glucose residues.

When the glucan was incubated with a purified exo-(1→3)-β-D-glucanase, isolated from the marine diatom *Chaetoceros affinis* var. *willei*[13], no detectable amounts of glucose were liberated, indicating that the (1→3) linkages were inaccessible to the enzyme.

The simplest possible structure for the glucan is **1**, but the evidence obtained does not exclude some variation in the length of the side chains.

TABLE II

ASSIGNMENTS OF PEAKS IN THE ^{13}C-N.M.R. SPECTRA (in p.p.m.)

Type of unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Intact glucan						
β-D-Glc	104	74	76–77	70–71	76–77	62
(1→3)-β-D-Glc			86–87			
(1→6)-β-D-Glc						69–70
Smith-degraded glucan						
β-D-Glc	104	74	77	71	76	70

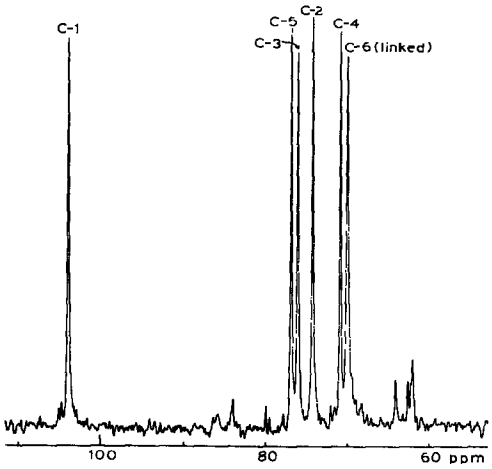
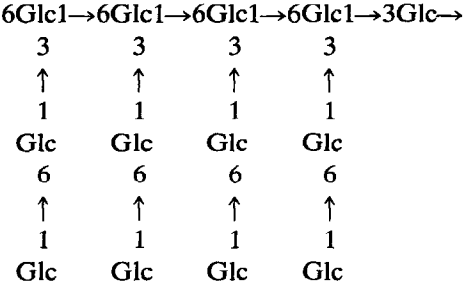


Fig. 2. ^{13}C-N.m.r. spectrum (25 MHz) of the Smith-degraded glucan.



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Thus, the reserve polysaccharide from *E. huxleyi* is a (1→6)-linked β-D-glucan with branching at positions 3, and (1→6) linkages in the side chains. Most of the structural investigations of β-D-glucans from related algae have shown that they are (1→3)-linked β-D-glucans, and branching at positions 6 has also been reported in glucans from other algae[14]. The glucan of the brown alga *Eisenia bicyclis*[15] consists of a linear sequence of (1→3) and (1→6) linkages in the ratio 2:1, but an essentially (1→6)-linked β-D-glucan with branch points at positions 3 has not previously been found.

EXPERIMENTAL

Culturing. — *Emiliania huxleyi*, clone C-hux, was isolated from off the Møre coast (Norway) by Dr. S. Myklestad. The cultivation and harvesting were carried out essentially as described[16] for *Skeletonema costatum*. A culture volume of 45 L

gave 2.5 g of freeze-dried alga, and extraction with 0.05M sulphuric acid (60 mL) gave, after neutralisation and dialysis, a pale-green powder (475 mg). This material was further purified according to the procedure of Nelson and Lewis[17], and dried *in vacuo* at 100° (yield, 404 mg).

Optical rotations. — Measurements were made at 20° with a Perkin–Elmer 141 polarimeter.

Reducing power. — The average d.p. of the glucan was determined by the measurement of reducing power[18] with D-glucose as the standard.

G.l.c. of alditol acetates. — The sample was hydrolysed in M H_2SO_4 at 100° for 5 h, and the alditol acetates were prepared as described by Blakeney *et al.*[19]. G.l.c. was performed on a Perkin–Elmer Sigma 2 gas chromatograph equipped with a flame-ionisation detector and a stainless steel column (2 m × 2.2 mm) of 1.5% of HI-EFF2BP on Chromosorb W.HP (100–120 mesh) operated at 190° or 155–190° at 1°/min. The carrier gas was nitrogen.

Methylation analysis. — The glucan was methylated by a modified Hakomori procedure[6]. A solution of the glucan (1 mg) in dimethyl sulfoxide (0.5 mL) was stirred with 2M methylsulfinylmethanide at room temperature for 20 h, and the reaction was monitored with triphenylmethane[20]. Methyl iodide (0.5 mL) was added and the mixture was stirred for 1 h. The methylated sample was purified[21] on a C_{18} Sep Pack cartridge by elution with $CHCl_3$–MeOH (1:1), then hydrolysed, reduced, and acetylated[7]. The products were analysed by (*a*) g.l.c. at 170° on a column (2.5 mm × 200 cm) filled with 3% of OV-225 on Varaport 30 fitted to a Varian 1440 gas chromatograph with a flame-ionisation detector and nitrogen as the carrier gas; (*b*) g.l.c.–m.s. using a Finnigan 4000 instrument fitted with a DB-5 fused-silica capillary column (0.25 cm × 30 m) with an initial injection temperature of 50°, followed by an increase to 200° at 4°/min.

Periodate oxidation and Smith degradation. — A solution of the glucan (64 mg) in water (30 mL) containing $NaIO_4$ (0.8 mmol) was stored overnight at room temperature. Ethylene glycol (800 μL) was then added and the polyaldehyde was reduced conventionally with sodium borohydride. After dialysis in tubing with a cut-off molecular weight of 1000, the polymer was partially hydrolysed with 0.5M sulphuric acid for 5 h at room temperature. The neutralised hydrolysate was applied to a column (1 × 30 cm) of Biogel P2 and eluted with water. The column was calibrated with lactose, stachyose, and Dextran PDT 5558 (mol. wt. 2280), a gift from Dr. K. Granath, Pharmacia. The fractions were assayed by the phenol–sulphuric acid method[22].

N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded at 80° on solutions in D_2O (external Me_4Si) with a Bruker WM-400 or JEOL JNM-FX 100 F.t. spectrometer. Spectra in which the n.O.e.s were removed were also measured, to ensure that relative peak areas represented relative abundances.

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