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CARBOHYDRATES OF THE ANTARCTIC BROWN SEAWEED *ASCOSEIRA MIRABILIS**†

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Key Word Index—*Ascoseira mirabilis*; Phaeophyta; alga; seaweed; carbohydrate; mannitol; laminaran; alginic acid; 'fucan'.

Abstract—Mannitol, sucrose and four monosaccharides were obtained from an ethanolic extract of *Ascoseira mirabilis*. Sequential extraction with aqueous calcium chloride, dilute acid and dilute alkali gave mixtures of laminaran, 'fucan' and alginic acid. Laminarans fractionated from the extracts contained different proportions of uniformly (1 → 3) and (1 → 6) linked chains of β-D-glucose residues. The 'fucan' contained varying proportions of fucose, galactose and glucuronic acid, small amounts of xylose, mannose, glucose, half ester sulphate and protein. Extraction of the weed under mild alkaline conditions gave a yield of 13.4% of low molecular weight calcium alginate with a mannuronate to guluronate ratio of 30:70 and only a small proportion of sequences of alternating residues. Selective extraction and fractionation gave alginate fractions rich (> 80%) in mannuronate or guluronate.

INTRODUCTION

Ascoseira mirabilis, a member of Ascoseiraceae (order Fucales, class Phaeophyta) is endemic to the Antarctic although it has migrated to other locations. The short stipes arising from the holdfast divide to produce long narrow blades. The whole plant is less than a metre in length and appears to be rarely exposed. No chemical studies have been reported previously.

RESULTS AND DISCUSSION

To facilitate the extraction of the carbohydrates the whole alga was first ground to a fine powder under liquid nitrogen and dried in a current of air. The quantities of the various carbohydrates are quoted as percentages of the dry weight of this powder.

Mannitol (0.9%) was recovered from an ethanolic extract. Its mp, mmp and the retention time of its peracetylated derivative on gas chromatography confirmed its identity. The residual syrup from the ethanolic mother liquor (0.4%) was shown by paper chromatography to contain sucrose, fructose, glucose, fucose and xylose. The presence of the three aldoses was confirmed by GC. The residual powder after ethanolic extraction was subjected to sequential extraction and the recovery and composition of the polymeric extracts separated are given in Table 1. PC and GC of hydrolysates of each extract gave the distribution of the various monosaccharides shown in Table 2. The powder (31%) remaining after the sequential alkaline extraction contained *ca* 1% of carbohydrate, comprising all the monosaccharides. It was discarded. The calcium chloride (1) and alkaline (3) extracts gave on fractionation on DE52-cellulose columns neutral and acidic material (Table 3).

Laminaran

The neutral reserve glucans of brown seaweeds comprise chains of (1 → 3)-linked β-D-glucopyranose residues having a number average DP of about 25, together with, in some cases, (1 → 6)-linked β-D-glucopyranose residues and (1, 3, 6)-branch points. The proportions of the latter two features show considerable species variation [2–6]. In addition a proportion of the chains may be linked glycosidically through their reducing terminal residues to one of the primary hydroxyl groups of D-mannitol.

In the present case neutral glucans (100% glucose) were obtained by ion-exchange chromatography of the calcium chloride, acid and alkaline extracts; the total yield was 0.9%. The glucan (1N) from the calcium chloride extract was subjected to a detailed analysis. The partially methylated glucoses obtained after two methylations are listed in Table 4; the results show a substantial proportion (38%) of (1 → 6)-linked residues and a low degree of branching (4%). On oxidation of glucan 1N with sodium metaperiodate 0.96 mole of oxidant was reduced and 0.40 mole of formic acid was released per mole of anhydro-unit, 60% of glucose was uncleaved and 70% of the original glucan was recovered as polyalcohol. The results are consistent with a high content of (1 → 6)-linked units as the amount of mannitol present is insufficient to account for the results. The proportion of unreacted [(1 → 3)-linked] units is in reasonable agreement with the methylation data. The ^{1}H and ^{13}C NMR data showed clearly that the glucan was composed principally of (1 → 3)- and (1 → 6)-linked β-glucopyranose residues. Signals arising from all the carbon (see Fig. 1) and most of the hydrogen (see Fig. 2) nuclei could be assigned by

*Part 7 in the series "Carbohydrates of Brown Seaweeds." For Part 6 see ref. [1].

†Dedicated to Dr. Naval Antia on the occasion of his retirement.

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Table 1. Recovery and composition of polymeric extracts from *Ascoseira mirabilis*

Extractant	Extract	Recovery (%)*	Carbohydrate (%)†	Uronic acid (%)‡	Protein (%)§
Hot aq. $CaCl_2$	1 H	2.7	53	44	0
Hot acid	2	1.6	51	50	5
Hot alkali	3	9.5	60	40	16
Total		13.8			

*Percentage of the dry wt.
†ManA:GlcA (1:1) standard graph.
‡ManA:standard graph and expressed as percentage of the carbohydrate.
§Percentage of the extract.

Table 2. Monosaccharide distribution in polymeric extracts from *Ascoseira mirabilis*

Extract	Monosaccharide distribution*					
	Fuc	Xyl	Man	Glc	Gal	Uronic acid
1 H	XX	X	X	XXX	X	XXXX
2	X	X	X	XXX	X	XXXX
3	XX	X	X	XXX	XXX	XXX

*Approximate relative amounts.

comparison with literature data [7–12]. Because each of the carbon and hydrogen nuclei resonate at one chemical shift, the (1 → 3)- and (1 → 6)-linked units must each have a single environment which persists largely throughout the whole polymer. These facts are consistent with either a single polymer containing interconnected sequences of uniformly (1 → 3)- and (1 → 6)- linked segments, or a mixture of two separate homopolymers containing these linkages. Gel permeation chromatography, which gave a single narrow peak corresponding to a DP of about 50 units, supports the former alternative.

Table 3. Composition of DE52 Column fractions from *Ascoseira mirabilis* extracts

Extract	Eluant	Fraction	Recovery (%)†	Carbohydrate (%)‡	Sugar distribution*							
					Fuc	Xyl	Man	Glc	Gal	ManA	GulA	GlcA
1	Water	1 HN	12	86				100				
	0.5 M KCl	1 HA	60	60	13	5	4	5	8	55	5	5
3	Water	3 N	28	85				100				
	0.1 M KCl	3A1	2	65				100				
	0.5 M KCl	3A2	40	60	20	11	5	3	24	20	5	10

*Neutral sugars determined by GC; mannose determined visually from paper chromatograms; uronic acids estimated by carbazole method.
†Percentage of the extract.
‡Percentage of the fraction.

Table 4. Methylated alditol acetates from *Ascoseira mirabilis* glucan 1N

Retention time*	Methylated sugar†	Linkage indicated	Molar ratio‡
0.97	2,3,4,6-Tetra-*O*-	Glcp-(1 →	3.4
1.82	2,4,6-Tri-*O*-	→ 3)-Glcp-(1 →	24.6
2.32	2,3,4-Tri-*O*	→ 6)-Glcp-(1 →	18.0
4.18	2,4-Di-*O*-	→ 3),→ 6)-Glcp-(1 →	2.0

*OV225 column, expressed relative to 2,3,4,6-tetra-*O*-Me-1,5-di-*O*-acetylglucitol as 1.00.
†2,3,4,6-Tetra-*O*- = 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol, etc. Confirmed by GC-MS.
‡Determined from peak areas and corrected for effective carbon response [18]. The figures are scaled to give a DP of about 50.

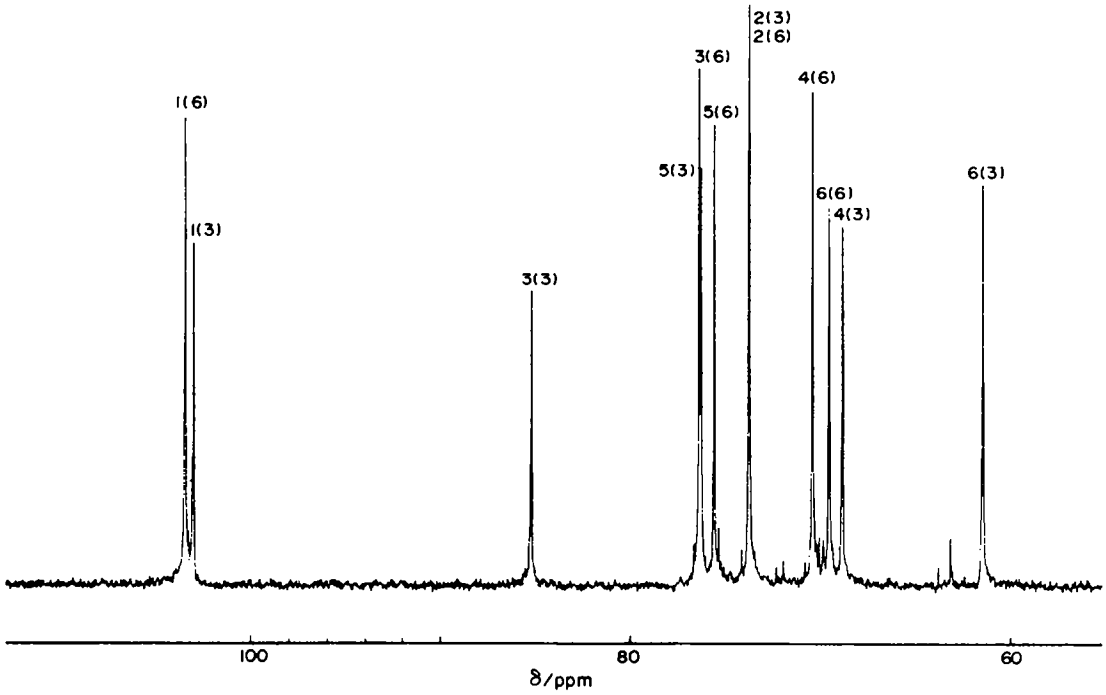

Fig. 1. 100.6 MHz ^{13}C NMR spectrum of *Ascoseira mirabilis* glucan 1N in D_2O at 75°. 1(3) signifies carbon atom 1 of a 3-linked glucosyl unit, etc.

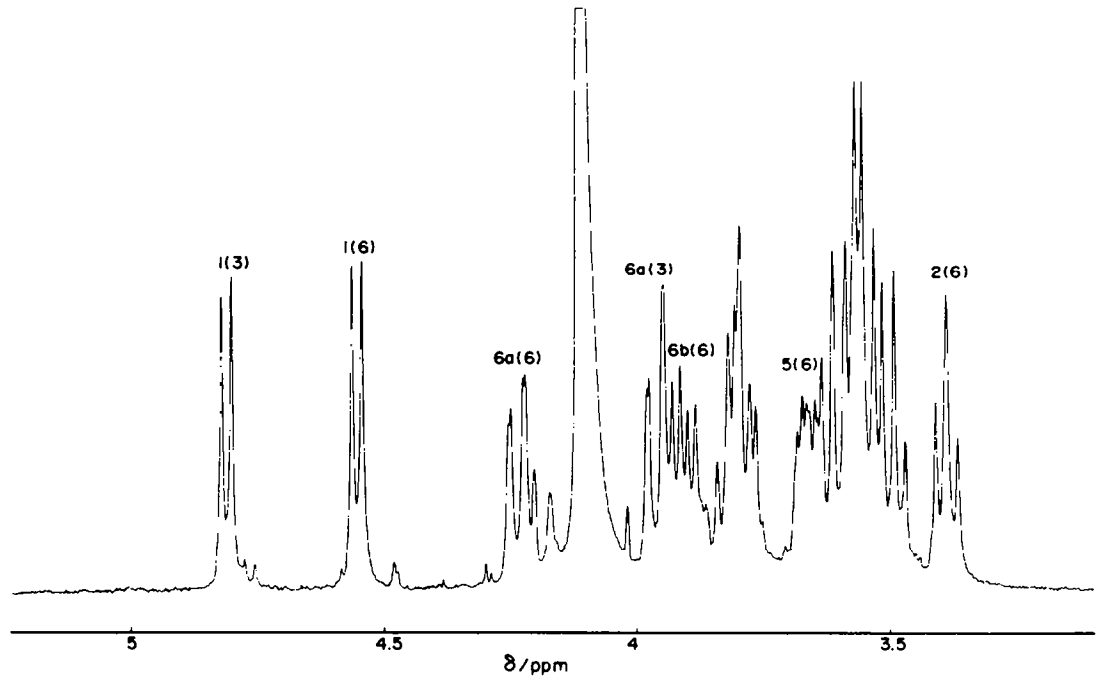

Fig. 2. 400.1 MHz ^1H NMR spectrum of *Ascoseira mirabilis* glucan 1N in D_2O at 75°. 1(3) signifies a proton on carbon atom 1 of a 3-linked glucosyl unit, etc.

Neutral β-glucans obtained from other extracts were also of the laminaran type but differed in detail. Thus aqueous calcium chloride at room temperature (instead of 70°) yielded a glucan (0.3%) with 75% of β-(1 → 3)- and 25% of β-(1 → 6)-linked units according to NMR spectroscopy. In addition the neutral component (3N) of the alkaline extract consisted of a β-glucan of higher apparent molecular weight than fraction 1N, and contained 40% of

(1 → 3)- and 48% of (1 → 6)-linked residues.

Taken together, the results suggest that *Ascoseira mirabilis* laminaran comprises a range of structures based on essentially linear chains of (1 → 3)- and (1 → 6)-linked β-D-glucopyranose residues. The susceptibility to extraction will depend not only on the proportion and arrangement of the differently linked residues, as noted previously [4, 13, 14] but also on the molecular size. The most commonly reported degree of polymerization, i.e. about 25 [15] corresponds to the limit for water solubility of a linear β-(1 → 3)-glucan [11]; the isolated *Ascoseira marabilis* glucans are somewhat larger than this. The degree of branching is much lower than that of *Cystophora* laminaran which also possesses a high proportion of (1 → 6)-linkages [6], two reports for *Eisenia bicyclis* laminaran are conflicting on this point [5, 16]. Non-covalent association of these glucans in the alga is to be expected from the *in vitro* behaviour of such structures [7, 11, 12] and their importance in the structures of cell walls of yeasts and fungi [17].

Fucans

Cellulose acetate electrophoresis at pH 5 of the two acidic fractions 1A and 3A2 of *Ascoseria mirabilis* from a DE52-cellulose column (Table 3) revealed a fast and a slow moving band for both materials. The former was assigned as 'fucan' and the slower band as alginic acid since at this pH the carboxyl groups of alginic acid are only partially ionised whereas the sulphate group in 'fucans' remains fully ionized. Preparative separation into two fractions of both of these materials was achieved by chromatography on Sephadex G100. From 600 mg of 1A, 200 mg of 'fucan' (1AF) and 300 mg of alginic acid (1AA) were isolated. Hydrolysates of the alginic acid contained only traces of neutral sugar. Ion exchange chromatography of 3A2 failed to give two distinct peaks. An arbitrary separation resulted in 83 mg of 'fucan' (3AF) and 181 mg of alginic acid (3AA). However hydrolysates of the latter showed substantial proportions of neutral monosaccharides indicating the presence of very low molecular weight 'fucan' as well as alginate. The composition of the two 'fucans' (1AF and 3AF) are shown in Table 5 and the monosaccharide composition in Table 6. Even with the difficulty of accurately quantifying the monosaccharides it can be seen that both 'fucans', but particularly 3AF, contain a large proportion of galactose. Galactose-rich 'fucans' are not usual although such have been reported previously for example in *Desmarestia aculeata* [19] and in *Sargassum linifolium* [20]. The presence of glucose in these 'fucans' is surprising although it has been reported previously as a constituent in the 'fucan' from *Padina pavonia* [21]. Elution of the present fucans from Sephadex 4B indicated an M_r range extending up to 5×10^5.

Table 5. Composition of *Ascoseira mirabilis* 'fucans'

Fraction	Carbohydrate (%)	Uronic acid (%)*	Sulphate (%)†	Protein (%)‡
1AF	62§	33	12	0
3AF	68‖	25	8	15

*Carbazole analysis.
†Expressed as a percentage of the carbohydrate.
‡Expressed as a percentage of the fraction.
§Fucose–galactose–glucuronic acid (1:1:1) standard graph.
‖Fucose–galactose–glucuronic acid–mannose (1:2:1:1) standard graph.

Alginic acid

Two alginic acid fractions were isolated during sequential extractions of *Ascoseira mirabilis* with (a) cold aqueous calcium chloride followed by anion exchange chromatography (alginate 1CAA) and (b) hot sodium carbonate followed by precipitation with calcium chloride (alginate 3AA). Direct ^{13}C and ^{1}H NMR analyses of these fractions (Table 7) showed that they were composed principally of polymannuronic and polyguluronic acid, respectively and according to solution viscosity and gel permeation chromatography were of low molecular weights equivalent to apparent DPs of about 20 and 40 units.

In order to circumvent the possibility of degradation during the sequential extractions, a sample of powdered alga was extracted directly with dilute alkali, and yielded 13.4% by weight of calcium alginate. This alginate showed no gel-forming property, and a 1% solution of the sodium salt had a relative viscosity of 2.0 Gel permeation on Sephadex G100 gave an elution profile similar to that of the sequentially extracted alginate, with an indicated average DP of about 100. The possibility of enzymic degradation during storage was considered, but analysis of fraction 3AA with thiobarbituric acid failed to reveal any 4,5-unsaturated residues which would be expected from lyase action [22]. Also the brown seaweed, *Macrocystis pyrifera*, stored under the same conditions, yielded alginate of high molecular weight. It is concluded that the alginate metabolized by *Ascoseira mirabilis* has a much smaller molecular weight than that generally found in the Phaeophyta. A low molecular weight alginate (DP 155, with antitumour properties) has also recently been reported in *Sargassum fulvellum* [23], and it would seem that in these species either the alginate cannot contribute to the mechanical properties of the algal tissue, or the contribution is not related simply to the property of gel formation as observed *in vitro*. Smidsrod and co-workers showed [24] that while the modulus of rigidity of algal

Table 6. Distribution of monosaccharide residues in fucan fractions

	Fuc	Xyl	Man	Glc	Gal	GlcA	ManA	GulA
1AF	26	8	7	9	17	23	8	2
3AF	15	8	12	8	27	15	10	2

Neutral sugars determined by GC; mannose determined visually from PC. Uronic acids estimated by carbazole method.

Table 7. Monomer compositions and distributions in *Ascoseira mirabilis* alginates by NMR spectroscopy

Alginate fraction	F_M	F_G	F_{MM}	F_{GG}	F_{MG}	F_{GM}	N_M	N_G
$CaCl_2$ aq., sequential (^{13}C)	0.84	0.16	0.59 (0.71)	0.15 (0.03)	0.16 (0.13)	0.10	6.6	1.2
Alkali, sequential (^{13}C)	0.14	0.86	0.18 (0.02)	0.74 (0.74)	0.06 (0.12)		2.4	15
Alkali, direct (^{13}C)	0.29	0.71	0.20 (0.08)	0.58 (0.50)	0.11 (0.21)		2.7	6.5
Alkali, direct (^1H)	0.32	0.68	0.26 (0.10)	0.62 (0.46)	0.06 (0.21)		4.3	11

Figures in parentheses are expected values for random distributions. F, Mole fraction; M and G, mannuronate and guluronate units, respectively; MM, GG, MG and GM, homo- and hetero-disaccharide diads; N_M and N_G, average chain lengths of homopolymeric sequences.

tissue is related to the alginate M/G ratio and cation composition, it is 100 times larger than that of a prepared gel, so other factors are clearly involved.

The results of the analysis of alginates by NMR spectroscopy given in Table 7 show, as expected, that the directly extracted material had a monomer composition and distribution similar to those of the sequential, alkaline extract. This latter fraction was thus enriched in polyguluronate by prior removal of the acid soluble fraction rich in mannuronate. The monomer distributions in all three fractions suggested that biosynthetic conversion of M to G residues is a non-random process and favours the generation of homopolymeric G blocks. The average G block length, N_{GG}, of the polyguluronate fraction indicates that a significant number of blocks approach the size required for Ca^{2+}-mediated association, i.e. *ca* 25 [25] however the *Ascoseira mirabilis* alginate does not form a mechanically stable gel. In principle only two junction zones per chain are required for the formation of a network, but in the present case the overall chain length is probably below the minimum required [26].

EXPERIMENTAL

Algal material. The sample of *Ascoseira mirabilis* was collected at Factory Cove, South Georgia, Antarctica, at a depth of 4 m in a water temp. of less than 10° on 4 February 1980 by members of the British Antarctic Survey. The alga was maintained at −20° from shortly after harvesting until it was ground under liquid N_2 to a fine powder and immediately extracted.

General methods. Uronic acid was determined by the *m*-hydroxybiphenyl method [27] or by the modified carbazole method [28], cellulose acetate electrophoresis was carried out as described in ref. [29]. Details of GC analysis and other general methods have been described previously [30, 31].

Extraction of carbohydrates. The dried powdered weed was extracted with 80% EtOH. After formaldehyde treatment [30] the residual material was first extracted with 2% aq. $CaCl_2$ at either room temp. or 70° (1), successive extractions were then made with dilute HCl at pH 2 (2) and 3% aq. Na_2CO_3 (3) both at 70°. In a large scale extraction 170 g of weed were extracted with 7 × 600 ml of each extractant for 4 hr. Alginic acid and 'fucan' in the alkaline extract were separated as previously described [30]. Alginic acid and 'fucan' were also extracted directly [32] from formaldehyde-treated weed (17.2 g) to yield calcium alginate (2.3 g) and soluble polysaccharide (1.68 g).

Examination and fractionation of the extracts. Each of the aq. $CaCl_2$ extracts was dialysed against frequent changes of deionized H_2O, concd and freeze-dried. The extracts were analysed for carbohydrate, uronic acid and protein contents. Portions were hydrolysed with 2 M TFA for 2 hr at 120°, and the hydrolysates examined by PC and by GC-MS as the alditol acetates. Portions of the extracts were fractionated on a column of DEAE-cellulose (DE52) by elution with H_2O followed by aq. KCl solns to yield neutral (N) and acidic (A) fractions (see Table 3). Neutral fractions were oxidized with 0.015 M sodium metaperiodate [31], reaction was complete after 96 hr; HCOOH released was measured by titration [33]. The alkaline extract was separated into Ca (II)-soluble ('fucan') and -insoluble (alginic acid) fractions; calcium alginate was converted to the sodium form by successive exchanges in 0.5 M HCl and 0.1 M NaOH [34].

NMR analyses. Samples for NMR analysis were deionised by passage through Dowex chelating resin Na^+ form, freeze dried and dissolved in D_2O or DMSO-d_6 (50 mg cm^{-3}). Spectra were measured at 400.1 (^1H) or 100.6 (^{13}C) MHz on a Bruker WH400 instrument using 5 mm or 10 mm probes at 343°K. Typical experiments used 32 k data points, spectral widths of 40(^1H) or 20 (^{13}C) kHz, 30–60° pulse angles, pulse repetitions of 3.4 (^1H) or 1.0 (^{13}C) sec, and 50–500 (^1H) or *ca* 50000 (^{13}C) scans. The solvent deuterium resonance was used as a field frequency lock and chemical shifts were expressed relative to external TMS. Alginate spectra were assigned according to refs [35–37].

The gelling properties of alginates were investigated by dropping 10% aq. solns of sodium alginates into 5% aq. $CaCl_2$; after 0.5 hr the drops were removed and examined.*

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