Phytochemistry, Vol. 22, No. 6, pp. 1429–1432, 1983.
Printed in Great Britain.

0031-9422/83/061429-04$03.00/0

CARBOHYDRATES OF THE BROWN SEAWEED *LESSONIA NIGRESCENS**†

ELIZABETH E. PERCIVAL, MARIO F. VENEGAS JARA and HELMUT WEIGEL

The Bourne Laboratory, Royal Holloway College, University of London, Egham, TW20 0EX, U.K.

(*Revised received* 26 *October* 1982)

Key Word Index—*Lessonia nigrescens*; Phaeophyta; seaweed; carbohydrates; alginic acid; 'fucan'.

Abstract—Stipes plus haptera and fronds were extracted separately. Crystalline mannitol and two oligouronic acids were separated from the ethanolic extracts of both parts of the weed. 'Fucans' were present in all the sequential aqueous, acidic and alkaline extracts. Each of the 'fucans' contained varying proportions of fucose (major), galactose, mannose, xylose, glucuronic acid, half ester sulphate and small proportions of protein. Fractionation of the aqueous extract failed to reveal any laminaran. Alginates were present in the sequential and direct alkaline extracts. The only significant differences between stipes and fronds were the higher proportion of glucuronic acid in the 'fucans' from the stipes and of the alginic acid in the stipes.

INTRODUCTION

The majority of chemical investigations carried out on the carbohydrates of seaweeds belonging to the Phaeophyta have been on extracts of whole weeds. It has, however, been shown [1] that in the alginates from *Laminaria digitata* the proportion of guluronic acid residues in the alginates increases through the series frond, stipe, unattached haptera, attached haptera. No similar studies have emerged for the 'fucans'. It was decided, therefore, to sequentially extract stipes plus haptera (hereafter called stipes) and fronds separately, as detailed earlier [2], and to investigate the respective extracts.

The brown seaweed, *Lessonia nigrescens*, is a member of the Lessoniaceae family and the order Laminariales. It grows abundantly in southern Chile and Argentina, normally on exposed shores, where it may occur from low water to a maximum depth of 38 m. Two other species are found in Chile and Argentina but *L. nigrescens* is distinguished from the others by its massive, solid holdfast (haptera) and numerous stipes, bearing long narrow fronds [3]. The only previous chemical studies on the carbohydrates of this genus were on *L. flavicans*, water soluble polysaccharides [4] from which 1.4% of a fucan was separated, and a report of the presence of alginic acid in *L. nigrescens* [5].

RESULTS AND DISCUSSION

Mannitol was recovered from the ethanolic extracts and recrystallized (140 mg from stipes and 81 mg from fronds, from 30 g of each). It had mp, and mmp, with a sample of authentic mannitol, of 165°, and the correct mobility on PC and retention time as the alditol acetate on GC. The ethanolic mother liquors from stipes and from fronds were shown by PC to be identical and were combined to yield a syrup (*ca* 200 mg). After separation on 3MM paper two oligouronic acids were isolated. The hydrolysates of both these materials contained fucose, xylose, mannose, galactose and glucuronic acid. The presence of these oligouronic acids could be due to degradation of 'fucans', but it is also possible that they are precursors of the macromolecules described later.

The weights and compositions of the different aqueous and acidic freeze-dried extracts are given in Table 1. It can be seen that the highest yields and carbohydrate contents were from the hot aqueous extractions, followed by those of the hot acidic extractions. It was decided, therefore, to confine further study to those two extracts, hereinafter known as 'fucans' A and B. From the sequential alkaline extract after removal of calcium alginate, 'fucans' DS, 230 mg from stipes, and DF, 250 mg from fronds, were isolated containing 62% and 42% carbohydrates, respectively. From direct alkaline extractions 'fucans' ES, 340 mg from stipes, and EF, 470 mg (51% carbohydrate) from fronds, were isolated as white powders.

The approximate molar proportions and percentages of the neutral monosaccharides in the hydrolysates of A, B and DF are given in Table 2. PC of hydrolysates of the other 'fucans' extracted by alkali showed the same sugars, with fucose as the major monosaccharide.

The total recovery of 'fucans' from the sequential extracts was *ca* 2.8% from stems and 2.6% from fronds, whereas by direct alkali extraction *ca* 7% and 9%, respectively, of 'fucans' were isolated.

The two 'fucans' BF and DF, both devoid of glucose, were found to contain this sugar after esterification and reduction. This could only be derived from glucuronic acid in the polysaccharides. Further proof of the identity of the uronic acid as glucuronic acid in 'fucan' EF was achieved by GC/MS (CI) analysis of the derived alditol acetates after reduction with $NaBD_4$. The ion with m/z 437 $[M+2]^+$, was present in the peak with the retention time of authentic glucitol acetate, conclusive proof of glucuronic acid in the 'fucan'.

Attempts to separate laminaran, the β-1,3-linked glucan present in most brown seaweeds, by elution of fucan A from a DE-52 cellulose column [6] with water failed. The

---

*Part 6 in the series "Carbohydrates of Brown Seaweeds". For Part 5 see ref. [6].

†A brief account of part of this work was presented at the Royal Society of Chemistry Carbohydrate Group Meeting held at Birkbeck College, London, in September 1981.

Table 1. Yields and constituents of the 'fucans' from the aqueous and acidic sequential extracts of 30 g samples

	Wt (mg)	Carbohydrate (%)*	Uronic acid (%)*	Sulphate content (%)*	Protein content (%)*
Stipes, cold water	110	47	19(40)	9(19)	7.3
Fronds, cold water	90	36	9(25)	10(28)	8
Stipes, hot water (AS)	224	62	20(32)	11(18)	10.3
Fronds, hot water (AF)	212	63	16(25)	13(20)	11.5
Stipes, cold acid	111	44	13(29)	10(23)	11.8
Fronds, cold acid	68	40	12(30)	9(22)	12.9
Stipes, hot acid (BS)	156	53	29(55)	6(11)	5.8
Fronds, hot acid (BF)	178	46	17(37)	7(15)	6.3

*Percentage calculated on weight of material.
Figures in parentheses calculated on carbohydrate content.

Table 2. Approximate relative molar proportion of the constituent monosaccharide sugars in the hydrolysates of the respective extracts calculated from GC traces of the alditol acetate derivatives

	Extract									
Sugars present	Stipes hot water (AS) (prop.)	(%)	Fronds hot water (AF) (prop.)	(%)	Stipes hot acid (BS) (prop.)	(%)	Fronds hot acid (BF) (prop.)	(%)	Fronds sequential alkali (DF) (prop.)	(%)
Fucose	5.2	60	3.2	43	4.55	63	6.7	82	4.5	57
Xylose	1.0	12	1.0	13	1.0	14	1.0	12	1.0	13
Mannose	1.4	17	1.6	22	0.7	10	0.5	6	0.7	9
Galactose	0.9	11	1.6	22	1.0	13	trace		1.7	21

aqueous eluate (3 % carbohydrate) was devoid of glucose. Subsequent elution with an increasing concentration of potassium chloride gave a similar pattern, that is decreasing uronic acid and increasing sulphate contents in the fractions, to those obtained for 'fucans' from other brown seaweeds [2, 6, 7]. Of the material applied to the column 97 % was recovered. The presence of laminaran is often dependent on the season of collection [1]. We have no means of verifying when the present material was harvested.

The presence of protein (Table 1) in all the freeze-dried solids isolated indicates that they may be proteoglycans. Fractionation of 'fucan' AF on Sepharose 4B and analysis for carbohydrate and protein gave the same elution pattern for the two polymers in the sense that carbohydrate and protein were present in all the fractions. However, covalent linkage between the two has still to be proved. The elution pattern of 'fucan' AF indicates polydispersity of the molecules, with a large portion of molecules with a relatively high M_r comparable with that of Dextran T500. The approximate M_r of 6.7×10^5 was obtained from the selectivity curve for Sepharose 4B using Dextrans T40, T70, T250, T500 and T2000 as standards [8].

'Fucan' A (SO_4^{2-} 10 %), after attempted alkaline desulphation [9], was recovered in 60 % yield without loss of sulphate. Analysis on a column of Sepharose 4B indicated a M_r of *ca* 8000 indicating cleavage of the glycosidic linkages during the treatment with alkali. Nevertheless, the retention of sulphate is evidence that those sulphate groups are not linked to carbon atoms which are adjacent to carbon atoms carrying free hydroxyl groups *trans* to the sulphate residues [10].

This is the first time that 'fucans' from fronds and stipes of a particular alga have been extracted separately and it is surprising that the two parts of the plants contain such essentially similar polymeric material soluble in water and in dilute acid. The only apparent difference is that the 'fucans' from the stipes have a higher uronic acid content (Table 1), particularly that from the hot acid extract where the uronic acid comprises 55 % of the carbohydrate.

Characterization of the alginates

From the sequential alkaline extraction calcium alginates were separated from the stipes (23.5 %) and from the fronds (16.5 %). By direct alkaline extraction calcium alginates from the stipes (41 %) and from the fronds (34 %) were obtained. All four materials were isolated as white powders. The higher yield by direct alkaline extraction is to be expected since it is known that the previous acid extraction in the sequential procedure preferentially removed mannuronic acid units from alginates [7]. In both extractions it can be seen that the yields from the stipes were higher than those from the fronds. PC of the hydrolysates of the alginates, from direct alkaline extraction of both stipes and fronds, showed the presence of both guluronic and mannuronic acids and this was confirmed by ionophoresis.

The relative viscosities of 1 % solutions of the sodium

Table 3. Assignments of peaks in the ^{13}C NMR spectrum of sodium alginate from *L. nigrescens*

Sequence	C-1	C-2	C-3	C-4	C-5	C-6
MMM	100.94	71.10	72.21	78.87	76.82	175.11
MMG	102.00	71.29	—	79.07	76.40	—
GMM	100.70	70.71	—	78.31	76.95	—
GMG	101.79	71.40	72.47	78.31	76.56	175.85
GGG	101.39	66.09	70.00	80.55	67.95	175.31
GGM	100.59	65.74	—	80.55	68.34	—
MGG	101.48	66.09	—	80.55	67.95	—
MGM	100.45	65.74	70.22	80.55	68.34	175.66

The C-6 resonances, due to asymmetric triads, have not been identified but probably most of them are similar to those from GGG and MMM sequences.

alginates obtained by direct alkaline extraction were low, that from the fronds 39 and 267 cps for that from the stipes.

The physical properties of alginates in the seaweed are strongly influenced by the proportions of mannuronic and guluronic residues in the macromolecules. In order to determine this, ^{13}C NMR analysis was carried out on the alginate obtained from the stipes by direct alkali extraction.

The assignment of the peaks was made following the conclusions of Grasdalen *et al.* [11] who studied whole alginates and fractions rich in different components. These authors found that the resonances given by the carbon atoms of the mannuronic and guluronic residues were modified by the residues on either side, so that the peaks can be assigned to triads of residues, where the signal is given by the central residue and is modified by those on either side of it. The assignment of the peaks in the present studies is given in Table 3 taking them in the same order as that given by Grasdalen *et al.* Although they are not exactly the same numerically to the published figures they are so close that this assignment is justifiable. Grasdalen *et al.* indicated that there could be some uncertainty about the assignment of the M peaks for C-2 and C-3. To obtain the M/G ratio, therefore, the peak areas for M and G residues for C-1, C-4 and C-5 were measured and their relative proportions (M/G) found to be 1.8.

EXPERIMENTAL

Algal material. The sample of *Lessonia nigrescens* investigated was harvested in Chile, South America and supplied to us by Alginate Industries Ltd. as dried material.

General methods. The general methods have been described previously [2]. Uronic acid was determined by the *m*-hydroxybiphenyl method [12] and by a modified carbazole method [13]. ^{13}C NMR analyses were carried out on a 400 MHz apparatus, at 353 K referenced to external TMS at 305 K. Protein was assayed by a protein-dye binding method [14].

Extraction of the carbohydrates. The dried powdered stipes plus haptera and fronds (30 g each) were subjected separately extractions with 80% EtOH [2]. After HCHO treatment the separate residual materials were extracted sequentially with 2% aq. $CaCl_2$, dil. HCl at pH 2 (first in the cold and then at 70° with both extractants) and finally with 3% aq. Na_2CO_3. The alginic acids and 'fucans' in the alkaline extracts were separated according to the methods previously described [2]. Alginic acid and 'fucans' were also extracted directly from the dried, HCHO treated, weed (stipes and fronds 5 g each), with alkali [6].

Examination of the extracts. Each of the extracts from stipes and fronds were dialysed against frequent changes of deionized H_2O, concd and freeze-dried.

After hydrolysis and separation on 3MM PC (solvent C) into neutral and acidic materials, the neutral fractions were reduced, converted into the acetate derivatives and analysed by GC on a 3% OV225 column linked to a mass spectrometer (EI and CI).

Characterization of the uronic acid. Aliquots (50 mg) of the dried 'fucans' (BF) isolated from the fronds by hot acid and (DF) by sequential alkali extraction were esterified under dry conditions with 3% HCl–MeOH. The derived esters were reduced with $NaBH_4$ and the reduced materials hydrolysed. The hydrolysates were analysed by PC in solvent C with glucose oxidase spray. The ester of the direct alkali extracted 'fucan' EF was reduced with $NaBD_4$, hydrolysed, reduced with $NaBH_4$ and the derived alditols were converted into their acetate derivatives and analysed by GC/MS (CI).

Molecular size determinations. An aliquot of 'fucan' AF was dissolved in H_2O (2 mg/ml) and layered onto a Sepharose 4B column (16.5 × 1.3 cm) and eluted with 1 M KCl. Fractions (2 ml) were collected and analysed for carbohydrate (1 ml) and assayed for protein (1 ml).

The alginates. The calcium alginates isolated by sequential extraction (7.64 g from stipes and 4.96 g from fronds, each from 30 g) and by direct alkali extraction (2.05 g from stipes and 1.71 g from fronds, each from 5 g) were converted into their respective Na salts [7]. Each of the sodium alginates were hydrolysed and analysed by PC (solvent C) and by paper ionophoresis in borax–$CaCl_2$ buffer, pH 8.9 [15]. The viscosities of 1% sols of direct alkali extracted alginates from stipes and from fronds were measured at 25° in an Ostwald capillary viscometer. The sodium alginate from the stipes was shaken twice with D_2O, freeze-dried and analysed by ^{13}C NMR spectroscopy.

Acknowledgements—We thank Alginate Industries Ltd., for the gift of the seaweed, and World University Services and Royal Holloway College for financial help to one of us (M.F.V.J.).

REFERENCES

1. Stockton, B., Evans, L. V., Morris, E. R., Powell, D. A. and Rees, D. A. (1980) *Bot. Mar.* **XXIII** 563.
2. Jabbar Mian, A. and Percival, E. (1973) *Carbohydr. Res.* **26**, 133.

3. Searles, R. B. (1978) *Br. Phycol. J.* **13**, 361.
4. Villarroel, L. H. and Zanlungo, A. B. (1975) *Rev. Latinoam. Quim.* **6**, 127.
5. Vargas Cobeña, R. H. (1974) *Bol. Soc. Quim. Peru* **40**, 55.
6. Percival, E., Rahman, Md., A. and Weigel, H. (1981) *Phytochemistry* **20**, 1579.
7. Carlberg, G. E., Percival, E. and Rahman, Md., A. (1978) *Phytochemistry* **17**, 1289.
8. (1971) *Gel Chromatography*. A laboratory manual, p. 15. Bio-Rad Laboratories California.
9. Rees, D. A. (1963) *J. Chem. Soc.* 1821.
10. Percival, E. and Wold, J. K. (1963) *J. Chem. Soc.* 5459.
11. Grasdalen, H., Larsen, B. and Smidsrod, O. (1981) *Carbohydr. Res.* **89**, 179.
12. Blumenkrauts, N. and Asboe-Hansen, G. (1973) *Analyt. Biochem.* **54**, 484.
13. Knutson, C. A. and Jeanes, A. (1968) *Analyt. Biochem.* **24**, 470; 482.
14. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
15. Haug, A. and Larsen, B. (1961) *Acta Chem. Scand.* **15**, 1395.