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Characterization of the alginates from algae harvested at the Egyptian Red Sea coast

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Dedicated to Professor H.S. El Khadem for the occasion of his 80th birthday

Abstract

The alginates from five species of brown algae from the Egyptian Red Sea coast, namely: *Cystoseira trinode, Cystoseira myrica, Sargassum dentifolium, Sargassum asperifolium*, and *Sargassum latifolium*, were isolated and their compositions and structures studied by ^{1}H NMR spectroscopy. All the alginates studied contain more guluronic acid (G) than mannuronic acid (M) and have a homopolymeric block-type structure ($\eta < 1$). The intrinsic viscosity of the alginate samples range from 8.6 to 15.2 and the gel strength ranges from 10.97 to 15.51. The constitutional G- and M-blocks of alginates from two different species (*C. trinode* and *S. latifolium*) were separated after partial acid hydrolysis. The ^{1}H NMR spectral data of the blocks GG and MM obtained by chemical fractionation were compared with those of polymeric alginates. The monomeric uronic acids were separated by complete acid hydrolysis of *S. asperifolium* alginate and the G and M monomers were characterized by ^{1}H, ^{13}C NMR spectroscopy as well as by paper electrophoresis.

Keywords: Alginate; Red sea algae; Guluronic acid; Mannuronic acid; ^{1}H NMR; Viscosity; Gel strength; GG and MM blocks

1. Introduction

The most well known polysaccharide of brown algae is alginate, a glycuronan of considerable commercial importance.[1] Alginate must be regarded as a collective term for a family of linear (1→4)-linked α-L-gulurono-β-D-mannuronans of widely varying composition and sequential structure.[2]

Alginate is located in the cell wall and in the matrix of the algae,[3,4] cementing the cells together and giving certain mechanical properties to the algae. In its native state, alginate exists as an insoluble mixed salt of all cations that are found in sea water,[5] the principal ones being sodium, magnesium, and calcium.[2] It is in rapid ion-exchange equilibrium with sea water. The extraction of alginate may thus, be regarded as a process in two steps: transformation of insoluble alginate into a soluble form namely sodium alginate, followed by diffusion of the soluble glycuronan into solution.[1,6]

^{1}H NMR spectroscopy[7] is suitable for characterizing both the composition and the distribution sequence of the two uronate residues in algal samples, and is particularly useful for quantitative work when only small amounts of material are available.

Studies of alginates[8–12] indicate that the two different uronic acid residues exist as blocks of homopolymeric sequences of either D-mannuronic acid residues (M-blocks) or L-guluronic acid residues (G-blocks), separated by long sequences of heteropolymeric matrial (MG-blocks), arranged in a nearly alternating fashion as shown here schematically:

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–M – M – M – M – M – M – … M – G – M – G – M – G – M – G – … G – G – G – G – G – G–
M-block MG-block G-block

The three types of blocks have been characterized by partial hydrolysis with HCl. The material thus solubilized corresponds to the MG-block. The resistant part is then fractionated at pH 2.9. The soluble fraction corresponds to the M-block, the insoluble to the G-block.[13]

For commercial and scientific purposes, the most important property of alginates is their ability to form viscous solutions in water, and thus alginate samples are usually characterized by means of their intrinsic viscosity.[14] The ability of alginates to form gels[15] in the presence of calcium ions is one of their main biofunctional properties, and is also of great industrial interest. The formation of gels depends mainly upon autocooperatively formed junctions between chain regions enriched in GG-sequences.[16,17]

2. Results and discussion

The dry ground brown algae used were analysed for dry weight and ash percentages (Table 1). High figures for ash were observed, probably because some of these specimens contained calcareous animals not be removed by extensive washing.

The brown algae were extracted with dilute hydrochloric acid, and the residue neutralized and then extracted with sodium carbonate solution. The intercellular nonbonded alginate was removed as the soluble sodium salt after treatment with acid and neutralization. The bonded alginate was obtained by extraction with sodium carbonate solution. The extracted alginate was recovered by precipitation with alcohol (Scheme 1).

The acid extract and the alcoholic solutions remaining after precipitation of alginate in both cases were analysed for total carbohydrates[6,18] and fucose[19] (Table 2).

The data from Table 2 indicate that the acid extract of *C. trinode* and *C. myrica* contain more carbohydrates than the other algal species, while the acid extract of *C. trinode* and *S. Latifolium* contain a higher amount of fucose. The alcohol-soluble part obtained from *S. dentifolium* after precipitation of alginate extracted by sodium carbonate also contained the highest amounts of carbohydrates. The five species studied contain fucose, a characteristic constituent of brown algae, and fucose-containing polysaccharides may, in a number of species, be the dominating polymeric constituent.

The alginate samples were examined by ^{1}H NMR spectroscopy and the monomeric composition, and the diad frequency determined according to the method of Grasdalen and coworkers[7] (Table 3).

The ^{1}H NMR spectra of whole alginate samples at neutral pD are shown in Fig. 1. It was necessary to degrade the samples to obtain well resolved signals.[7] The relative areas of the peaks A(G-1), B(M-1 and GM-5), and C(GG-5) contain information on the uronic acid composition and the fractions of nearest neighbours along the copolymer chain. Quantitatively, the mole fraction of G and the doublet frequency F_{GG} are related to the intensities (I) of the respective lines by the following relationships:

$$F_G = \frac{I_A}{I_B + I_C};$$

$$F_{GG} = \frac{I_C}{I_B + I_C}$$

The mole fraction of M is then derived from the normalization condition

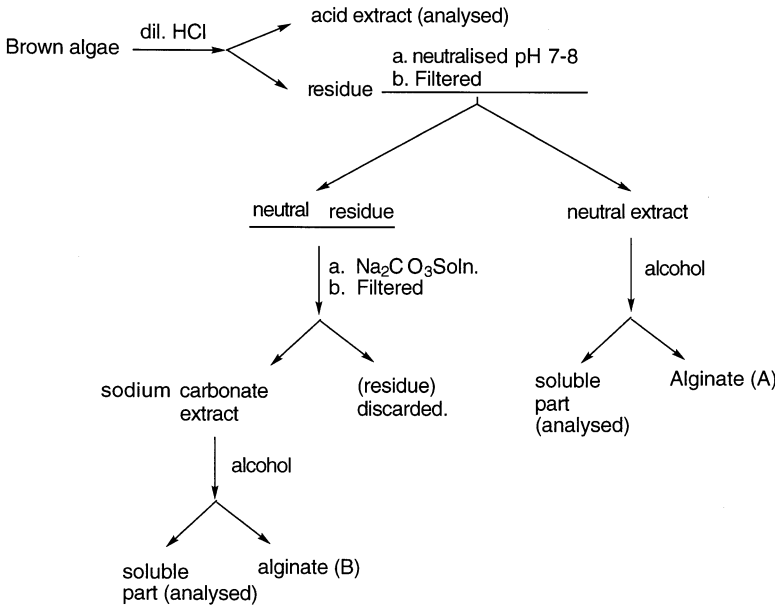
$$F_G + F_M = 1$$

The relations between the mole fractions and the doublet frequencies are given by

Table 1
Algal species

Sample	Weight (g)	Dry weight (%)	Ash (%)	Organic matter (%)	Yield of alginate (g)	Algal residue (g)
C. trinode	5	96.94	61.44	35.50	0.054 [a] 0.163 [b]	1.885
C. myrica	5	99.28	81.65	17.63	0.110 [b]	1.73
S. dentifolium	5	97.92	71.58	26.34	0.164 [b]	1.896
S. asperifolium	5	97.67	54.98	42.69	0.605 [b]	1.669
S. latifolium	5	97.24	44.57	52.67	0.859 [a] 0.215 [b]	1.242

[a] Alginate extracted in the neutral step.
[b] Alginate extracted by Na_2CO_3.



Scheme 1.

$F_{GG} + F_{GM} = F_G$; $F_{MM} + F_{MG} = F_M$

For long chains (DP $n > 20$), and corrections for the reducing-end residues may be neglected, so that $F_{MG} = F_{GM}$. Hence, numerical values for the uronic acid composition and for the doublet frequencies may be calculated (Table 3).

The alginate isolated from *C. trinode* and *S. dentifolium* exhibited a dominant A peak (Fig. 1(a and c)), in accord with its high mole fraction of G. About one-half of the M residues occured in alternating sequences. The alginate from *C. myrica* contained more G than M (Fig. 1(b)). The alginate from *S. asperifolium* and *S. latifolium* (sodium carbonate extract and neutral extract) (Fig. 1(d–f)) contained higher M than the foregoing samples.

A complete description of alginate monomer sequence is not possible by the presently determined doublets only. However, it may be illustrative to use a parameter η, defined by the equation:

$$\eta = \frac{F_{MG}}{F_M \times F_G},$$

to characterize and test the sequence distributions.

The studied Red Sea alginate samples, showed η in the range 0.16–0.56 (Table 3), which suggests[7] a structure of the homopolymeric block type ($\eta < 1$).

2.1. Determination of intrinsic viscosity

Vincent and coworkers[20]prepared alginates from some Canadian brown algae, and observed intrinsic viscosities varying from 3.6 to 19.2. The intrinsic viscosity of the alginate samples studied here ranges from 8.6 for *C. trinode* and *C. myrica* to 15.2 for *S. asperifolium* (Table 4).

2.2. Determination of gel strength

The addition of salts of divalent metals to sodium alginate solutions lead in most cases to increased viscosity followed by precipitation. Haug[6] found that such a viscosity increase is indicative of gel formation, and that different divalent metals have different gel-forming abilities; these increase as the affinity of alginate for the divalent ion increases. The gel strength was determined by a standard procedure.[15] The gel strength of the alginate samples studied, ranges from 10.97 for *C. myrica* to 15.51 for *S. asperifolium* (Table 5).

2.3. Partial acid hydrolysis

Alginates from two different species of brown algae (*C. trinode* and *S. latifolium*) were partially hydrolyzed[13] with 0.3 M hydrochloric acid and the results are given in Tables 6 and 7. Table 8 gives the results of block determination of alginates from the two species using phenol–sulfuric acid method.[18] The amounts of the three types of blocks varied considerably. The table, shows that, in *C. trinode*, the percentages of MG- and GG-blocks are close, whereas the MM block is lower. However, the GG block in *S. latifolium* is nearly triple the MG value.

^{1}H NMR results of alginates of *C. trinode* and *S. latifolium* may be compared with those obtained by chemical fractionation.[21] Ideally, the latter method should separate the partly degraded alginate molecule into an M-block, consisting only of MM doublets in

Table 2
Total carbohydrate determination during the alginate extraction steps

Sample	Mg sugar/100 mg organic matter								
	Alginate content of the algae, mg/100 mg organic matter		Acid extract [c]		Alcohol-soluble part (neutral step) [d]		Alcohol-soluble part (Na_2CO_3 extraction step) [e]		
	Alginate [a]	Alginate [b]	Total sugar	Fucose	Total sugar	Fucose	Total sugar		Fucose
C. trinode	3.04	9.18	74.93	11.63	12.58	1.10	4.40		0.77
C. myrica		12.48	72.39	5.38			19.58		0.83
S. dentifolium		12.45	57.87	5.62			50.97		0.56
S. asperifolium		28.34	32.16	4.15			7.84		1.11
S. latifolium	32.62	8.16	42.26	8.24	14.40	≅ 0	7.64		0.61

[a] Alginate extracted in the neutral step.
[b] Alginate extracted by Na_2CO_3.
[c] HCl acid extract of the treated algae.
[d] The residual algae after treatment with HCl, filtered and the precipitate suspended in water, neutralized, filtered and the filtrate concentrated, precipitated with alcohol and the alcohol soluble part analysed.
[e] The remaining mother liquor after alcohol precipitation of alginate extracted by Na_2CO_3 was concentrated and analysed.

NMR terminology, a G-block consisting only of GG doublets, and a soluble fraction (MG-block) enriched in MG and GM doublets (Table 9).

The results in Table 9 are in agreement with the foregoing observation that there are several sources of error. One of these is the solubilization of the homopolymeric blocks during the partial hydrolysis, which affects the MM blocks more than the GG blocks.[22] This is clearly observed in Table 9, where the MM blocks of *C. trinode* and *S. latifolium* obtained by chemical fractionation are much lower than those obtained from ^{1}H NMR spectroscopy.

From the ^{1}H NMR spectral measurements[23] for M- and G-blocks of *S. latifolium* [Fig. 2(a–c)], it was found that the M-block fraction constitutes of 85% of M, and the G-block fraction contains 88% of G (Table 10). In *C. trinode,* (Fig. 2(d)) the G-block fraction contains 96% of G (Table 10).

2.4. Complete acid hydrolysis and separation of the M- and G-monomers

Haug and Larsen[24] described a method for quantitative determination of the uronic acid composition of alginates, based on hydrolysis with 80% sulfuric acid at 20 °C, followed by treatment with 2 M acid at 100 °C, and then separation of the uronic acids in the hydrolysate by chromatography on an anion-exchange column. The loss of uronic acids in the different steps in the procedure has been investigated, and a factor correcting for the difference in the rate of breakdown of guluronic and mannuronic acids is introduced. After acid hydrolysis of *S. asperifolium* alginate with sulfuric acid and separation of the uronic acids from the hydrolysate by column chromatography using anion-exchange resin,[24] guluronic acid was eluted first from the column followed by the mannuronic acid fractions, (Fig. 3). The amounts of the different uronic acids were determined by the phenol–sulfuric acid method.[18]

Using the standard procedure[25] and multiplying the proportion between mannuronic and guluronic acids by 0.66, the correct factor,[24] gave an estimate of the uronic acid composition of the alginate sample used (0.69) (Table 11).

$$\%\ (\mathrm{Gul}+\mathrm{Man}) = \frac{74.40 + 77.96}{500} \times 100 = 30.5\%$$

$$M/G = \frac{77.96}{74.40} = 1.05$$

$$M/G_{\mathrm{correct}} = 1.05 \times 0.66 = 0.69.$$

The M/G ratio (0.69) obtained by hydrolysis is in full agreement with the ratio ($M/G = 0.41/0.59 = 0.69$) obtained by ^{1}H NMR measurements of the polymeric *S. asperifolium* alginate.

Table 3
Compositions, doublet frequencies, and the block character (η) in alginate samples

Alginate sample	Composition, fractions		Doublet frequencies				η
	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}	
C. trinode [a]	0.37	0.63	0.24	0.13	0.13	0.50	0.56
C. myrica [a]	0.31	0.69	0.21	0.10	0.10	0.59	0.47
S. dentifolium [a]	0.34	0.66	0.23	0.11	0.11	0.55	0.49
S. asperifolium [a]	0.41	0.59	0.30	0.11	0.11	0.48	0.45
S. latifolium [a]	0.45	0.55	0.41	0.04	0.04	0.51	0.16
S. latifolium [b]	0.42	0.58	0.34	0.08	0.08	0.50	0.33

[a] Sodium carbonate extract.
[b] Neutral extract.

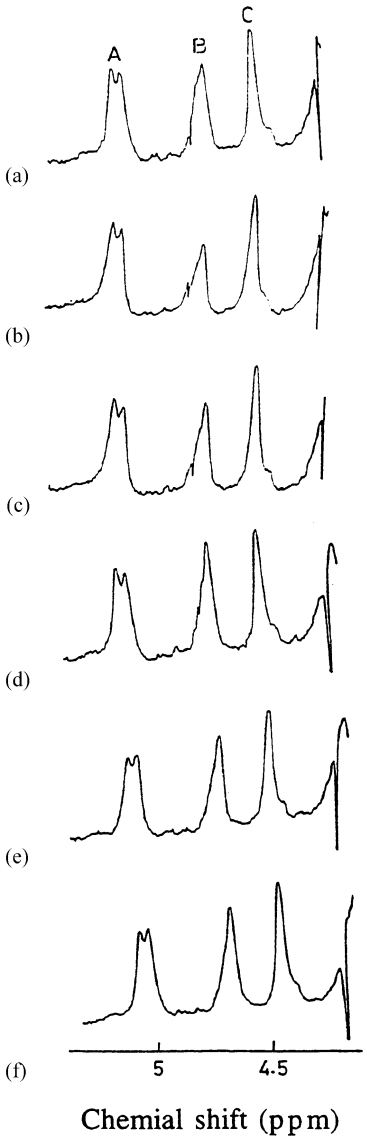


Fig. 1. ^{1}H NMR spectra (100 MHz) of the algal sodium alginates of (a) *C. trinode*, (b) *C. myrica*, (c) *S. dentifolium*, (d) *S. asperifolium*, (e) *S. latifolium*, and (f) *S. latifolium* (neutral extract), in D_2O.

Table 4
Absolute viscosity, relative viscosity, and intrinsic viscosity of alginate samples η_o (0.1 M NaCl) = 99

Alginate sample	$\eta_{sol.}$	$\eta_{rel.}(\eta_{sol.}/\eta_o)$	Intrinsic viscosity [η] dL/g
C. trinode	380.89	3.84	8.6
C. myrica	381.626	3.85	8.6
S. dentifolium	266.49	2.69	12.6
S. asperifolium	320.52	3.23	15.2
S. latifolium	393.782	3.97	8.7

It is noteworthy that the amounts of guluronic and mannuronic acids obtained by column separation are in agreement with the calculated values from phenol–sulfuric acid (Table 11).

2.5. Characterization of guluronic acid by ^{1}H NMR spectroscopy

The 400-MHz ^{1}H NMR spectrum (Fig. 4) of a solution of sodium L-GulA shows the same spectral pattern of strong peaks reported[25] for methyl β-D-gulopyranosiduronic acid.[26] A large $J_{1,2}$ value (9.0 Hz) and a collapse of the H-3 and H-4 resonances were observed in each spectrum. The large $J_{1,2}$ value indicates H-1 and H-2 to be *trans*-diaxial and implies that in the L configuration, the guluronate molecule exists in the $^1C_4$ (L) conformation, (Fig. 4) mainly as the β-pyranose form in aqueous solution.[26]The less shielded equatorial H-1 of the α-pyranose form resonates at 5.10 ppm ($J_{1,2}$ 3.0 Hz). The tautomeric equilibrium for L-GulA in aqueous solution also shows one furanose form. Its H-1 resonance at 5.18 ppm ($J_{1,2}$ 3.0 Hz) is attributable to a β-furanose ring.[27] Stereochemical effects favour this 1,2-*trans* β anomer strongly over the corresponding 1,2-*cis* α-anomer.

The proton-decoupled 100-MHz ^{13}C NMR spectrum of a solution of sodium L-GulA in D_2O (Fig. 5) shows one strong and one weak set of resonances, corresponding to the pyranose anomers. The C-2 and C-5 peaks are identified by comparison with the spectrum of the β-

Table 5
Gel strengths of the alginate samples

Alginate sample	Weight	Height	Diameter	Load (g) ($G_{meas.}$)	$G_{corr.}$	Mean gel strength (N/cm^2)
C. trinode	3.0272	1.94	1.38	229	16.1	
	2.9835	1.94	1.35	232	16.7	15.47
	2.7085	1.96	1.28	206	13.6	
C. myrica	2.8521	1.94	1.34	167	11.0	
	2.5009	1.92	1.24	160	9.5	10.97
	2.9109	1.96	1.32	173	12.4	
S. dentifolium	2.9065	1.96	1.33	251	17.70	
	2.7279	1.93	1.32	243	15.14	15.31
	2.5618	1.96	1.27	218	13.10	
S. asperifolium	2.8994	1.96	1.35	240	16.46	
	2.8338	1.94	1.33	259	17.37	15.51
	2.4231	1.85	1.28	253	12.69	
S. latifolium	2.5002	1.84	1.28	228	12.14	
	2.8308	1.90	1.36	250	15.74	14.67
	2.8845	1.91	1.36	245	16.14	

pyranose in solution.[25] The L-guluronopyranose ring predominates in the $^1C_4$ (L) conformation in solution, in accord with published data.[26] The α-L-guluronic acid (α-L-GulA) is the most important constituent in the brown algal polysaccharide.[25] An X-ray study[28] of sodium α-L-GulA dihydrate indicated an α:β ratio ~9:1.

2.6. Characterization of guluronic and mannuronic acids by paper electrophoresis

Paper electrophoresis was carried out according to the modified procedure[29] of Haug and Larsen for separation of uronic acids. For quantitative determination, the method has the advantage that the alkaline buffer solution automatically transforms all lactones to uronic acid salts.

Table 7
Total carbohydrate analysis by the phenol–sulfuric acid method of the M- and G-blocks obtained from *C. trinode* and *S. latifolium* alginates by partial acid hydrolysis

Sample	Total carbohydrate (mg)	
	C. trinode	*S. latifolium*
Soluble material	176.82	83.84
Insoluble material (M- and G-blocks)	263.73	331.82
Soluble material (M-block)	57.69	62.07
Insoluble material (G-block)	167.46	245.69
After refractionation of G-block		
Soluble material	3.35	5.35
Insoluble material (G-block)	151.96	129.49

3. Experimental

3.1. General methods

The ^{1}H NMR spectra were recorded on a Jeol FX 100 FT NMR spectrometer at 100-MHz with Me_4Si as the internal standard. The UV spectra were recorded on a UV-260 UV–visible recording spectrophotometer. The gel strength was calculated by using a Stevens-Lfra Texture analyzer. The viscosity (η) was determined by a Ubbelohde viscometer (Schott Gerate AVS 310). The pH was determined by using a pH meter (Orion Research Microprocessor pH/mili-voltmeter 811). The

Table 6
Weights (g) of M- and G-blocks obtained from *C. trinode* and *S. latifolium* alginates by partial acid hydrolysis

Alginate sample	Weight (g) M-block (soluble)	G-block (insoluble)	*M*/*G*	After refractionation of G-block	
				M-block	G-block
C. trinode	0.071	0.155	0.458	0.005	0.091
S. latifolium	0.087	0.169	0.515	0.003	0.1

Table 8
Block distribution of alginates from *C. trinode* and *S. latifolium*

Alginate sample	Partial hydrolysis [a]			*M*/*G* ratio [b]
	Soluble (%)	Insoluble (%) Soluble at pH 2.9	Insoluble at pH 2.9	
C. trinode	40.14	15.34	44.52	0.34
S. latifolium	20.17	16.10	63.73	0.25

[a] For 2 h with 0.3 M HCl at 100 °C.
[b] Using phenol–sulfuric acid method.

Table 9
Comparison between block distribution and doublet frequencies for alginate samples from *C. trinode* and *S. latifolium* (MM = M-block; GG = G-block)

Sample	MG+GM [c]	MM	GG	*M*/*G* ratio
C. trinode [a]	0.26	0.24	0.50	0.48
[b]	0.40	0.15	0.45	0.33
S. latifolium [a]	0.08	0.41	0.51	0.80
[b]	0.20	0.16	0.64	0.25

[a] Doublet frequency calculated from ^{1}H NMR.
[b] Block distribution obtained by chemical fractionation.
[c] Acid-soluble fraction by the chemical method.

samples were centrifuged on a Sorvall RC-5B refrigerated super-speed centrifuge. The freeze-drier used was Lyovac GT 2 Ley, Bold-Heraeus. Electrophoresis was carried out with an LKB power supply type 3371 B using Scheicher and Schull 2043b paper strips.

3.2. Algal samples

The five algae samples studied were: *C. trinode, C. myrica, S. dentifolium, S. asperifolium,* and *S. latifolium*. The samples were collected from the Red Sea area, washed, air dried, kept overnight at 30 °C in oven and ground to pass a 20 mesh-screen. The ground materials were stored in plastic bags. The dry weights were determined after drying overnight at 105 °C and the ash percentages were determined after burning the samples overnight at 400–450 °C.

3.3. Extraction of alginate

The extraction of alginate was carried out according to the method of Haug[6] and Larsen.[30] The ground algal material was treated with 0.5 part of formaldehyde (37% GR) before extracting with fifty parts of 0.2 M HCl. The residue obtained was suspended in 100 parts of distilled water and the pH of the suspension was maintained between 7 and 8. After filtration, NaCl was added to the

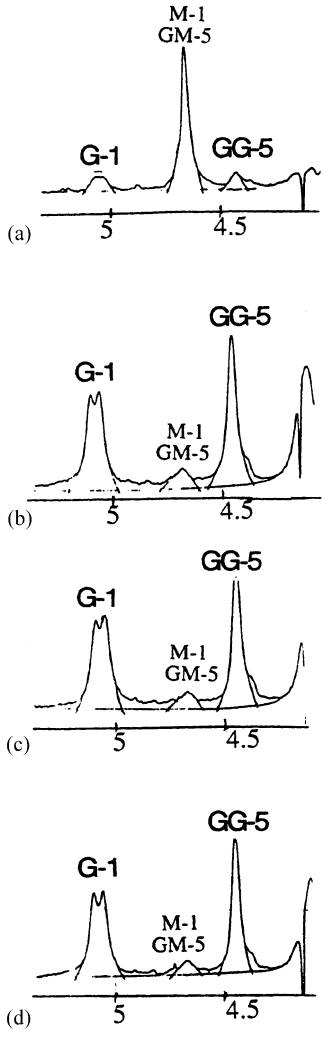


Fig. 2. (a) Expansion of the low field area of M-block of *S. latifolium* alginate; (b) expansion of the low field area of G-block of *S. latifolium* alginate; (c) expansion of the low field area of G-block of *S. latifolium* alginate after refractionation; (d) expansion of the low field area of G-block of *C. trinode* alginate after refractionation.

Table 10
The composition the doublet frequencies, and the block character (η) in the blocks of alginates

Alginate sample	Composition, fractions		Doublet frequencies				η
	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}	
S. latifolium							
M-block	0.85	0.15	0.82	0.03	0.03	0.12	0.24
G-block	0.12	0.88	0.07	0.05	0.05	0.83	0.47
G-block after refractionation	0.14	0.86	0.13	0.01	0.01	0.85	0.08
C. trinode							
G-block after refractionation	0.04	0.96	$\cong 0.0$	0.08	0.08	0.88	2.08

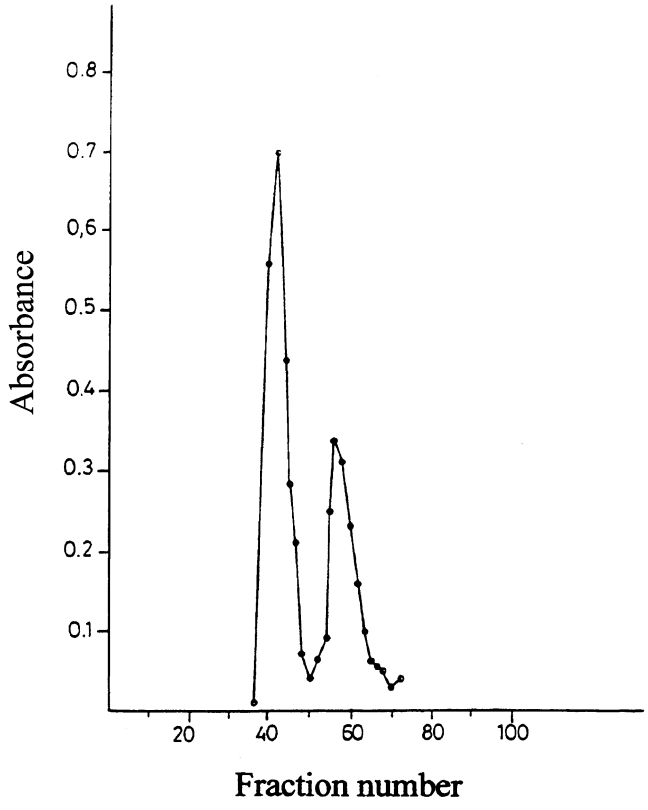


Fig. 3. Phenol–sulfuric acid test of the hydrolysate fractions containing material after complete acid hydrolysis of *S. asperifolium* alginate by sulfuric acid.

Table 11
Guluronic/mannuronic acid composition of *S. asperifolium* alginate, by weight and by the phenol–sulfuric acid method

Uronic acid component	Weight (mg)	Carbohydrates by phenol–sulfuric acid method (mg)
Guluronic	78	74.40
Mannuronic	80	77.96

filtrate to a 1% concentration, and the alginate was precipitated by adding to the solution an equal volume of EtOH. The residue from the filtered mixture was extracted with 50 parts of 3% Na_2CO_3, filtered and the filtrate was dialysed, and concentrated under vacuum to a low volume. Sodium chloride was added to a 1% concentration and, then the alginate was precipitated with EtOH.

The yields for the five samples are given in (Table 1). The residues from the samples were also determined after heating overnight at 105 °C.

3.4. Determination of reducing carbohydrates by the phenol–sulfuric acid method

The phenol–H_2SO_4 reaction[6,31] was carried out as follows: 2 mL of a solution, containing between 6 and 60 μg carbohydrate, was mixed with 0.5 mL 3% aqueous phenol in a test tube. Blank duplicates were included by using 2.0 mL distilled water. Concentrated H_2SO_4 (5 mL) was added rapidly from a pipette, the mixture immediately stirred with a glass rod, and kept for 30 min. The tube was cooled in an ice–water bath and the optical density recorded at 485 nm (Table 2).

$$\text{Number of mg carbohydrate} = \frac{\text{reading} \times f \times c}{0.1} \times \text{volume of the sample},$$

where f is a factor, 0.0137 corresponds to an alginate content of 0.0137 mg/mL in the test sample with an alginate containing 60% M.

c = concentration in 2 mL (0.5 solution × 4 or 1 mL × 2)

3.5. Determination of fucose[19]

The alginate solution (1 mL) was withdrawn into a test tube cooled in an ice–water bath (1.0 mL distilled water was used for blank). Sulfuric acid solution (4.5 mL) [made by adding carefully 600 mL concentrated H_2SO_4 to 100 mL distilled water and cooled to room temperature (rt)] was added and the tube was allowed to stand for ~1 min. While the tube was kept in an ice–water

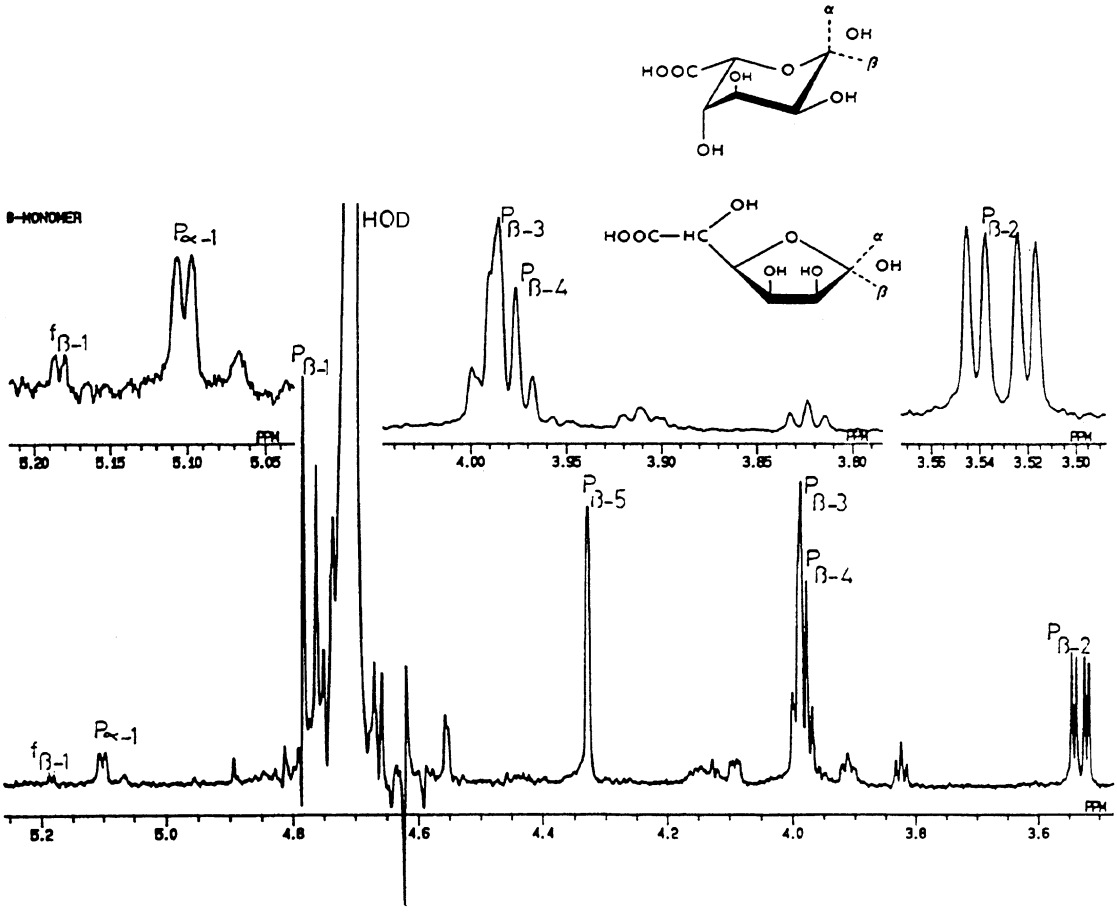


Fig. 4. 400-MHz, ^{1}H NMR spectrum of L-guluronic acid of *S. asperifolium* alginate, in D_2O.

bath, the contents were mixed. The tube was then transferred to a boiling-water bath, boiled for 10 min, and then cooled immediately to rt. Cysteine solution (0.1 mL) (3% w/v solution in distilled water) was added and mixed well. After 30 min the optical density at 396 nm and at 427 nm was measured. The optical density due to fucose is OD_{396}-OD_{427} (Table 2).

$$\text{Number of mg fucose} = \frac{\text{reading} \times f}{0.1} \times \text{volume of the sample}$$

where f is a calibration factor, 0.006, factor obtained by analyzing a sample of pure L-fucose and is corrected for the removal of one molecule of water in the formation of a polymer.

c = concentration in 1 mL (0.5 solution × 2)

3.6. Preparation of the alginate sample for doublet frequencies by NMR spectroscopy

To diminish the viscosity of the alginate solutions, they were partly degraded[7] by very mild acid hydrolysis. For alginates showing low solubility in acid media, as polymers rich in G- and/or M-blocks, the solutions ($\sim$0.4%) were treated, under constant stirring, with HCl solution until a pH of 5.2 was reached. The samples were then boiled under reflux for 1 h. After cooling to rt, HCl was added to pH 3.7 and the solutions were again boiled under reflux for 1.5 h. After cooling, the solution was neutralized by NaOH solution. The samples were freeze-dried, and then 10 mg of the residual sample was dissolved in 0.4 mL D_2O and 0.02 mL of triethylenetetramine hexaacetic acid (TTHA) was added to the solution. The solution was kept overnight and then used for NMR (Table 3).

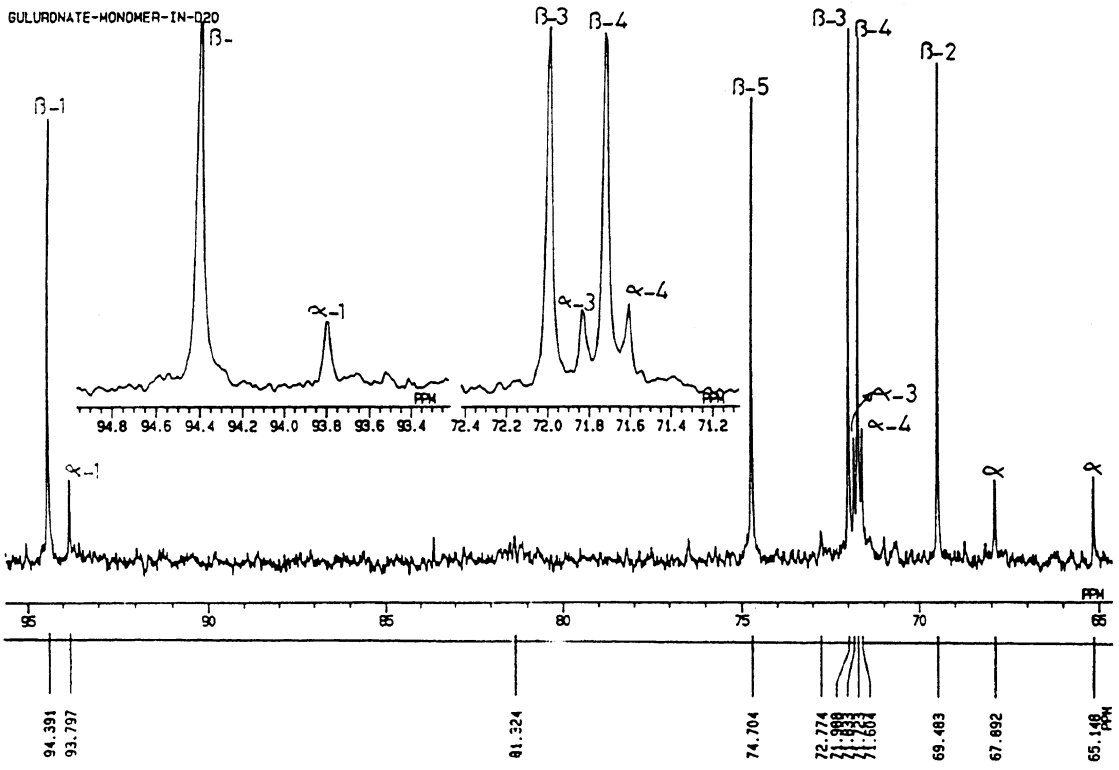


Fig. 5. Expansion of the 100-MHz ^{13}C NMR spectrum of L-guluronic acid of *S. asperifolium* alginate, in D_2O.

3.7. Determination of intrinsic viscosity

The measurements were carried out at 20 °C according to the method of Haug and coworkers[14] A 0.2% alginate concentration in 0.1 M NaCl solution was employed. The absolute viscosity of the alginate solution was recorded. The relative viscosity is given by

$$\eta_{rel} = \frac{\eta_{sol}}{\eta_o},$$

where η_{sol} is the absolute viscosity of the alginate solution and η_o is the viscosity of the solvent (0.1 M NaCl). For the standard samples, the η_{rel} values were plotted against the corresponding intrinsic viscosity $[\eta]$ at different alginate concentrations. Finally, the intrinsic viscosity $[\eta]$ was found by extrapolating to the alginate concentration in standard curves.[13]

3.8. Determination of gel strength

Gel cylinders[15] were made by placing aqueous solutions of sodium alginate (3%) containing 0.2 N NaCl in plastic cylinders (diameter = 1.27–1.37 cm, height = 1.84–1.95 cm) covered with cellophane membrane. Each sample was then placed in a beaker containing 0.2 M NaCl and 0.1 M $CaCl_2$ for 48 h. After removing the gel plug from the plastic cylinder, it was carefully dried on water-absorbent paper and weighed. Gel strength was determined as the force necessary to compress the alginate gel cylinders by 2 mm with a speed 0.2 mm/s. After that, correction was made for the gel strength (Table 5).

3.9. Partial acid hydrolysis and fractionation of M- and G-blocks

This followed the method of Haug and coworkers,[13] by suspending one part of alginate (0.5 g) in 100 parts of 0.3 M HCl. After 2 h at 100 °C, the suspension was cooled and centrifuged. The amount of material in the solution was determined by the phenol–H_2SO_4 method.[18] The residue was washed with 0.01 M HCl, suspended in water, and dissolved by careful neutralization. The volume was adjusted to give an alginate concentration of 1%, NaCl was added to 0.1 M, and the solution was adjusted with 0.025 M HCl to pH 2.8–3.0. The precipitate was collected by centrifugation, washed with 0.01 M HCl, suspended in water, and dissolved by neutralization. The amounts of carbohydrate in the precipitate after neutralization and in the supernatant solution were determined by the phenol–H_2SO_4 method. The two fractions were filtered off, dialysed

overnight, and freeze-dried. The G-block separated as a precipitate and M-block as supernatant. This experiment was carried out for two species of alginates (*C. trinode* and *S. latifolium*).

3.10. Refractionation of the G-block

The G-block fraction (100 mg) was dissolved in water to give a concentration of 1%, NaCl was added to 0.1 M, and the solution was adjusted with 0.025 M HCl to pH 2.8–3.0 then treated as before to separate the G-block as a precipitate and the M-block as the filtrate.

3.11. Preparation of samples for doublet frequency measurements by ^{1}H NMR spectroscopy

D_2O (0.4 mL) was added to 10 mg of the sample and 0.02 mL of triethylenetetramine hexaacetic acid, (TTHA) was added, and the solution was kept overnight and then measured.

3.12. Complete acid hydrolysis and separation of M- and G-monomers

Sodium alginate of *S. asperifolium* (500 mg) was mixed with 5.0 mL of 80% H_2SO_4[24] and the mixture was cooled in an ice bath, kept for 18 h, then the temperature was raised gradually to rt. The acid was diluted to obtain an acid strength of 2 M by adding 65 mL of water with cooling in an ice bath. The mixture was then heated in a boiling-water bath in sealed glass tube for 5 h. The tube was cooled, opened, and the mixture neutralized with slight excess of $CaCO_3$. The precipitate was filtered off and washed with water until about twice the original volume of hydrolysate has passed through the filter paper. The hydrolysate was concentrated to low volume and applied to a column (20×1.8 cm) of Dowex 1×8 anion-exchange resin, OAc^- form (200–400 mesh). Before applying the hydrolysate to the column, all lactones were transformed into uronic acids. Gradient elution was performed with 0.5–2 M AcOH and fractions ($\sim$15 mL) were collected. Uronic acids were detected out by dipping the filter paper in a solution containing 2.5% aniline trichloroacetate in glacial AcOH, drying at rt, and then heating for 3–5 min at 105 °C. The spots were detected under UV light.

Tubes containing material were tested by the phenol–H_2SO_4 assay.[18] L-Guluronic acid was eluted first from the column, fractions 36–48 and, D-mannuronic acid was eluted later,[31] fractions 53–70. Identical fractions were collected and evaporated to dryness, and traces of AcOH were removed by coevaporation with water. Neutralization was carried out by using 2 M NaOH (pH 6–8) and the samples were freeze-dried.

3.13. Preparation of samples for characterization of uronic acids by NMR spectroscopy

A sample from the guluronic acid component was prepared for ^{1}H NMR spectroscopy and another one for ^{13}C NMR spectroscopy. For ^{1}H NMR, a 5 mg sample was dissolved in 0.6 mL of D_2O and 10 μL of triethylenetetramine hexaacetic acid, (TTHA) was added. For ^{13}C NMR, 25 mg of guluronic acid was likewise dissolved in 0.6 mL D_2O at pD 7 and measured at 45 °C. A sample of mannuronic acid was prepared for ^{1}H NMR by dissolving a 5 mg sample in 0.6 mL of D_2O.

3.14. Characterization of guluronic and mannuronic acids by paper electrophoresis

The modified procedure[31] of Haug and Larsen for separation of uronic acids was used. The buffer solution used composed of 0.01M borax solution (pH 9.2) containing 0.005M $CaCl_2$ which gave 2 h a satisfactory separation of the four uronic acids. Authentic galacturonic and glucuronic acids (1% solution in H_2O and EtOH, 1:1) were commercial samples, whereas mannuronic and guluronic acids were samples earlier isolated. A solution containing 2.5% anilinetrichloroacetate in glacial AcOH was used for detection of samples. The strips were dipped in this reagent, dried at rt, heated for 3–5 min at 105 °C and the spots were detected under UV light.

4. Conclusion

The studied alginates of Red Sea brown algae showed high mole fraction of G and a homopolymeric block-type structure. The viscosity of alginates ranges from 8.6 to 15.2. The gel strength ranges from 10.97 to 15.51. The algae studied contain an appreciable amount of fucose. The constitutional MM and GG blocks were isolated by partial hydrolysis and identified by ^{1}H NMR and chemical fractionation for *C. trinode and S. latifolium*. The mannuronic acid (M) and guluronic acid (G) residues were separated by complete acid hydrolysis of *S. asperfolium* alginate. The observed *M*/*G* ratio of 0.69 obtained by complete acid hydrolysis was the same as that obtained by ^{1}H NMR spectroscopy and is within the range observed for a large number of brown algae (see Ref. 2). The L-guluronic acid monomer has the $^1C_4$(L) conformation.

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