Structural studies on agar fractions extracted sequentially from chinese red seaweeds: *Gracilaria sjeostedtii*, *G. textorii* and *G. salicornia* using ^{13}C -NMR and IR spectroscopy

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Editor's note

A conference on West Pacific Circulation Influence in China Seas (WEPACICS) was held during November 10-14, 1986 in Qingdao, China, under the joint auspices of the Institute of Oceanology, Academia Sinica (IOAS) and the National Science Foundation, United States, and under the sponsorship of Ya Hsueh, Florida State University, and Hu, Dunxin, IOAS. The primary subject of the conference was the influence of the West Pacific Circulation in the Yellow Sea and the East China Sea through the intermediary of the Kuroshio.

In the conference more than 20 papers were presented, summarizing the works on the interaction between the Yellow Sea and East China Sea, and oceanic circulation. Research experiences gained in the studies of the Gulf Stream and its influences in the U. S. coastal waters were shared.

In order to facilitate scientific exchange we have already published four papers by Hu Dunxin, Y. Hsueh, Su Jjian and Wang Wei, L. P. Atkinson and T. J. Berger in Vol. 5 No. 4 and by Guan Bingxian in Vol. 6 No. 1. In this and succeeding numbers, we will publish more of this conference's papers under the WEPACICS heading

STRUCTURAL STUDIES ON AGAR FRACTIONS EXTRACTED
SEQUENTIALLY FROM CHINESE RED SEAWEEDS:
GRACILARIA SJOESTEDTII, *G. TEXTORII* AND *G.*
SALICORNIA USING ¹³C-NMR AND IR SPECTROSCOPY*

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Abstract

The polysaccharides of three species of Chinese *Gracilaria* (Rhodophyta) were extracted by sequential solvent extraction and the chemical structures of the fractions obtained were investigated by using ¹³C-NMR and IR spectroscopy.

The ¹³C-NMR spectra showed that the main extract, cold water fraction, from *G. sjostedtii* consisted of repeating disaccharides of agarobiose and (1→3)-β-D-galactopyranosyl 1-(1→4)-α-L-galactopyranose 6-sulfate, the latter being a precursor to agarobiose and converted to agarobiose after alkali modification. The component of the 60% ethanol fraction from *G. textorii* was composed mainly of agarose 6-sulfate, 6-O-methylagarose and some amount of agarose, whose structures remained unchanged after alkali treatment. The autoclave extract from *G. salicornia* revealed a complex structure composed of 6-O-methylagarose, 2-O-methylagarose, branched 4-O-methyl-L-galactose and agarose 4-sulfate. All the substituents in these agaroses were stable against alkali treatment. The alkali extracts from *G. sjostedtii* and *G. textorii* showed the distinct spectra of amylopectin-like floridean starch, while that from *G. salicornia* showed a mixture of agaroses and floridean starch.

From the IR spectra the relative contents of total sulfate, 3,6-anhydro-galactose, 4-sulfate and 6-sulfate in some fractions of polysaccharides of the algae were qualitatively estimated by the ratios of absorption bands of 1250/2920, 930/2920, 850/2920 and 820/2920, respectively.

I. INTRODUCTION

It is well known that the cell wall polysaccharides of red seaweed mainly consist of galactan. Among them agar-type polysaccharides are the long chain molecules alternating with the repeating disaccharide agarobiose, (1→3)-β-D-galactopyranosyl-(1→4)-3,6-anhydro-α-L-galactopyranose. The hydroxyl groups in the two sugar residues may be substituted by methoxyl, sulfate and/or pyruvate to some extent (Fig. 1 B). Some red seaweeds have L-galactose 6-sulfate sugar residue alternating with D-galactose residue in the polymer chain (Fig. 1 A), which is considered as a biological precursor to agarobiose.

The agars from *Gracilaria* species are reported in highly heterogeneous structures with various substituents in the polysaccharide molecules [9,10,15]. Recently, agar was fractionated on the DEAE-

* Contribution No. 1489 from Institute of Oceanology, Academia Sinica

Fig.1 Disaccharide repeating units of agar

A: Biological precursor to agaroligosaccharides

- (1) $R_1 = R_2 = R_3 = H$, agaroligosaccharide, (1 → 3)-β-D-galactopyranosyl-(1 → 3)-6-ethylidene-α-L-galactopyranoside
- (2) $R_1 = R_3 = H$, $R_2 = CH_3$, 6-O-methylagaroligosaccharide
- (3) $R_1 = CH_3$, $R_2 = R_3 = H$, 2-O-methylagaroligosaccharide
- (4) $R_1 = R_2 = CH_3$, $R_3 = H$, 2,6-di-O-methylagaroligosaccharide
- (5) $R_1 = R_2 = H$, $R_3 = SO_3$, agaroligosaccharide 4-sulfate
- (6) $R_1 = R_3 = H$, $R_2 = SO_3$, agaroligosaccharide 6-sulfate
- (7) $R_1 = H$, $R_2 = CH_2COOH$, $R_3 =$ agaroligosaccharide 4,6-pyruvate

Sephadex A50^(5,10) or Dowex 1⁽⁸⁾ column to separate the neutral portion from the charged portions with different charge density. The fractions obtained were submitted to chemical analysis, enzymic degradation and ¹³C-NMR spectroscopic study for elucidating their chemical structures^(5,9,10).

In the present study, sequential solvent extraction⁽¹⁵⁾, ¹³C-NMR and IR spectroscopic methods were applied to investigate the structure of the fractions of agars extracted from *Gracilaria* *spestedtii*, *G. textorii* and *G. salicornia*, collected along the coast of China.

II. MATERIALS AND METHODS

1. Materials

Gracilaria spestedtii Kylin and *G. textorii* (Sur.) De-Toni were collected in Qingdao, northern China, in November of 1983 and December of 1985, respectively. *G. salicornia* (C.Ag.) Dawson was collected on Hainan Island, southern China, in April of 1984. All the samples were sundried without freshwater washing and stored in plastic bags.

2. Sequential solvent extraction⁽¹⁵⁾

10 g of algal pieces milled with MICRO-MILLTM to 1 mm in length were extracted twice with water at 22°C (1 l each, shaken overnight), and then the algal residues were successively refluxed thrice with boiling 95%, 80%, 60% and 40% ethanol solution (300 ml for 2 hours each), extracted thrice with boiling (hot) water (300 ml for 1 hour each), autoclaved twice with water at 121°C (300 ml each, 3 hours and 15 minutes), extracted twice with 8 mol/L urea solution at 80°C (100 ml each, 2 and 1 hour), and with alkali solution (1 mol/L NaOH) at 80°C (100 ml each, overnight). The extracts of each solution were combined, centrifuged (10,000 rpm), evaporated *in vacuo*, dialyzed, press

filtered through 1.2 μm and 0.45 μm filter membranes, and then freeze-dried. Some extractions in three algae were omitted (see Fig. 2).

Fig.2 Distribution pattern of fractions extracted from *Gracilaria spestedtii* (A), *G. textorii* (B), and *G. salicornia* (C). CW: cold water extract; 80, 60, 40: 80%, 60% and 40% ethanol extracts; HW: hot water extracts (1 and 2 refer to 1st and 2nd extraction); HA: weak acid extract; U: urea extract; AL: alkali extract; AU: autoclave.

3. ¹³C-NMR and IR spectroscopy

¹³C-NMR spectra were recorded with 4-5% solutions at 80°C of polysaccharides in D₂O using a Bruker WH 400 spectrometer at 100.62 MHz. The chemical shifts were measured in ppm relative to internal dimethylsulfoxide (DMSO) and converted to values relative to external tetramethylsilane (TMS). IR spectra of polysaccharide films were recorded on a Perkin-Elmer Infrared 297 Spectrophotometer.

4. Alkali modification^(1,5)

130 mg of polysaccharides were dissolved in 30 ml water in a 100 ml Erlenmeyer flask. 30 mg of NaBH₄ were added to the solution, and heated at 80°C for 15 minutes. After 15 ml of 3 mol/L NaOH solution were added, it was heated again for 3 hours at 80°C, and then extensively dialyzed against water and freeze-dried.

III. RESULTS

1. Fractionation pattern

Three species of *Gracilaria* tested exhibited quite different fractionation patterns. The cold

Table 1 ^{13}C -NMR Chemical shifts of carbons of repeating disaccharides in fractions isolated from *Gmattaria* spp. by sequential extraction (ppm, relative to TMS)

Species	Factions	C (1,3-linked D-glucose)						A (1,4-linked D-glucose)							
		1	2	3	4	5	6	1	2	3	4	5	6		
<i>Carrutaria spiosidilli</i>	Cold water extract	C	102.4	70.2	82.2	68.8	75.4	61.4	A	98.3	69.9	80.1	77.4	75.6	69.4
	Cold water extract (alkali-treated)	C	102.4	70.2	82.2	68.7	75.4	61.4	A	98.3	69.9	80.1	77.3	75.6	69.4
	Hot water extract	C	102.4	70.2	82.2	68.8	75.4	61.4	A	98.3	69.8	80.1	77.3	75.6	69.1
	Hot water extract	C*	103.5	69.9		69.1	75.8	61.7	A*	98.3	69.9	80.1	77.4	75.6	69.1
	Hot water extract	C	102.5	70.2	82.5	68.8	75.5	61.3	C	100.1	72.2	73.8	78.0	71.9	61.3
	Hot water extract	C*	103.5	69.9		69.1	75.8	61.7	A*	101.1	70.8	78.7	67.7	67.5	69.1
<i>Carrutaria textorii</i>	Cold water extract	C	102.4	70.1	82.2	68.8	75.4	61.5	A	98.3	69.9	80.2	77.4	75.7	69.1
	Cold water extract	C	102.5			69.0	73.6	71.8	A	98.3	69.9	80.2	77.4	75.7	69.1
	Cold water extract	C	102.4	70.1	82.2	68.8	75.4	67.4	A	98.3	69.9	80.2	77.4	75.7	69.1
	Cold water extract	C*	102.5			69.0	73.6	71.8	A	98.3	69.9	80.2	77.4	75.7	69.1
	Cold water extract	C*	102.5			68.4	73.0	67.5	A	98.3	69.9	80.2	77.4	75.7	69.1
	Cold water extract	C	102.4			68.4	73.0	67.5	A	98.3	69.9	80.2	77.4	75.7	69.1

[illegible]

C₁, C₂, C₃, C₄, C₅ and C₆ refer to the carbons of D-galactose, D-galactose of the precursor to agarobiose, 6-OCH₃-D-galactose, D-galactose of 2-OCH₃-D-galactose, D-galactose of the branched L-galactose and D-glucose of 2-OCH₃-L-galactose and L-galactose (sulfate), respectively; A, B and A' to the carbons of 3,6-anhydro-L-galactose, L-galactose 6-sulfate and 2-OCH₃-L-galactose, respectively; B to the carbons of the branched L-galactose; C and C' to the carbons of D-glucose of D-glucose, respectively; T: 4-OCH₃ in L-galactose.

water fraction of *G. sjostedtii* (Fig. 2A) gave the highest yield (12.7%), followed by 60% ethanol (9.6%), 40% alkali (7.9%), 40% ethanol (4.1%), urea (3.6%), and hot water fractions. The yield of the alkali fraction was the highest in the three species of *Gracilaria* studied. The total yield of fractions on dry basis of the algae amounted to 41.2%.

In the case of *G. textorii* (Fig. 2B) the 60% ethanol extract was the main fraction, yielding 14.6% of the algae, followed by 40% ethanol (6.7%), hot water (4.6%), urea (3.8%) and alkali (2.8%) extracts. The yield of other fractions was around 1% or less. The total yield of fractions of the algae was 36.3%.

The fractions from *G. salicornia* (Fig. 2C) revealed an interesting pattern different from two other species of *Gracilaria* above-mentioned. The yield of the cold water and all ethanol extracts was low, around 1%, but when the temperature was raised to 121°C (autoclave) the polysaccharide associated with the cell wall was released and dissolved into the solution, amounting to 18%. The hot water and alkali extracts were only 4.3% and 2.6% in yield, respectively, while the other fractions yielded much less. The total yield of fractions was 32.1% of the algae.

2. ¹³C-NMR spectroscopic analysis

The ¹³C-NMR chemical shifts (ppm) of carbons of disaccharide repeating units in fractions obtained from three species of *Gracilaria* by sequential extraction are summarized in part in Table 1, and those of various agaroses reported in literature are listed in Table 2 for comparison.

Gracilaria sjostedtii: The ¹³C-NMR spectra of the fractions from *G. sjostedtii* are shown in Fig. 3 and their chemical shifts in Table 1. But the chemical shifts of 60% ethanol, alkali-treated 60% and 40% ethanol extracts were not listed, since the first one is similar to that of the cold water extract and the latter two to the alkali-treated cold water extract. The spectrum of the cold water extract (Fig. 3A, a), a major fraction of *G. sjostedtii*, gave 12 clear signals for agarose (G1-G6, A1-A6) and characteristic signals: G*1-G*6, A*1-A*6 (Table 1, Table 2) for D-galactosyl-L-galactose 6-sulfate (Fig. 14), a precursor to agarobiose. The latter chemical shifts disappeared after alkali treatment, and only the agarose signals remained (Fig. 3Ab, since L-galactose 6-sulfate was transformed to 3,6-anhydro-L-galactose (Fig. 3A, a→b). The relative content of precursor to agarobiose in the cold water extract as estimated using the ratio of integrals of signals at A*1 and A1 shown in Table 3

Table 2 Chemical shifts of carbons of repeating disaccharide units of various agaroses (ppm, relative to TMS)

Repeating disaccharide units	1	2	3	4	5	6	OCH ₃
Agarobiose ^[19]	G 102.3	70.1	82.2	68.6	75.2	61.3	
A 98.2	69.7	80.0	77.2	75.5	69.7		
6-O-methyl-agarobiose ^[20]	G' 102.4	70.1	82.2	68.6	73.6	71.8	59.1
2-O-methyl-agarobiose ^[19]	G'' 102.6	70.2	82.7	68.7	75.6	61.4	
A'' 98.7	78.8	78.4	77.6	75.3	69.8	59.1	
Agarobiose-6-sulfate ^[20]	G* 102.3	69.9	81.9	68.3	72.8	67.5	
Precursor to agarobiose ^[20]	G* 103.7	70.5	81.1	69.1	75.9	61.8	
A* 101.2	69.8	71.0	79.0	67.7	67.5		

* Illustrations are the same as in Table 1

Fig. 3 ^{13}C -NMR spectra of agar fractions extracted from *G. spirostictii*.

A: (a) cold water, (b) cold water (alkali-treated), (c) 40% ethanol, (d) hot water extracts.

B: (a) urea, (b) alkali extracts.

G and A refer to the carbons in D-galactose and 3,6-anhydro-L-galactose of agarobiose, respectively; G* and A* refer to agarobiose; C to the D-galactose and L-galactose of precursor to agarobiose; C to the carbons D-glucose in floridean starch, and C' to its branch.

following the formula $\frac{A^*1}{A^*1 + A1} \times 100\%$ was 33.2% of total agaroses, decreased to 10.2% in 60% ethanol extract, and to nothing in 40% ethanol extract. This indicated that the precursor to agarose is more hydrophilic. The chemical shifts attributed to the amylopectin-like floridean starch C1-C6 (Fig. 3d, Table 1) were observed in addition to the signals for agarose and weak signals for the precursor in the spectrum of the hot water extract. As shown in Fig. 3Ba, the spectrum of urea extract gave more distinct signals of floridean starch and that of alkali extract showed almost pure

Table 3 Contents of various agaroses in fractions estimated by the ratio of integrals of signals in ^{13}C -NMR spectra

<i>Gracilaria spirostictii</i>			<i>Gracilaria textorii</i>			<i>Gracilaria salicornia</i>		
Fraction	A*	n of total agaroses	Fraction	n of total agaroses	G*	G'	Fraction	n of total agaroses
Cold water	33.2		Cold water	11.0	19.8		40% ethanol	91.9
40% ethanol	10.2		40% ethanol	27.8	31.7		60% ethanol	86.2
40% ethanol	Agarose		40% ethanol	46.8	25.3		40% ethanol	80.8
			Hot water	39.1	25.8		Antiarose	88.5
			60% ethanol	41.6	25.3			
			(alkali-treated)					
			40% ethanol	37.3	33.0			
			(alkali-treated)					

* Illustrations are the same as in Table 1.

floridean starch. C1-C6 with the branches C', coinciding with those of amylopectin B reported by Dais et al.^[4] (Fig. 3Bb), where C1 and C4 denote the carbons of C1 and C4 at the non-reducing end of D-glucose residue in the side chain as shown in Fig. 4.

Fig. 4. Floridean starch

Gracilaria textorii: All the NMR spectra of the cold water, hot water, 60%, 40% ethanol and alkali-treated 40% ethanol extracts (the latter two are not shown in Table 1) revealed resonance signals of the carbons of agarobiose (G1-C6, A1-A6), of the carbons of 6-O-methyl-agarose (G5, G'6, OCH₃) and of the carbons of D-galactose 6-sulfate (G⁺1, G⁺4, G⁺5, G⁺6) (Fig. 5, Table 1). The relative contents of these three agaroses were estimated by the ratio of the integrals of G6, G'6 and G⁺6 (as shown in Table 3) following the formula $G' \text{ (or } G^+) = \frac{G'6 \text{ (or } G^+6)}{G'6 + G^+6 + G6} \times 100\%$. The D-galactose 6-sulfate (G⁺), which was more hydrophilic and much more soluble in the cold water fraction, amounted to 41%, while 6-O-methyl-D-galactose (G'), which was more hydrophobic and much more soluble in higher concentration of ethanol (60%), amounted to 32% of the total agaroses. The

chemical shifts of all these three components after alkali modification were unchanged, indicating the sulfate and methyl groups on C6 of D-galactose are alkali-stable (Table 1). The alkali extract from *G. textorii* gave the NMR spectrum of floridean starch C1-C6, quite similar to that of *G. sinvestedtii* (Table 1). In addition, two unknown signals 77.6 and 77.8 ppm were present in the spectra of the cold water, 60% and 40% ethanol and hot water extracts, even after alkali treatment of the extracts (Fig. 5).

Fig. 5 ^{13}C -NMR spectra of agar fractions from *G. textorii*

(a) cold water, (b) 60% ethanol, (c) 60% ethanol (alkali-treated) extracts.

G' and A' refer to carbons in 6-O-methylagarulose;

G* and A* to carbons in agarulose 6-sulfate; G, A and C are the same as in Fig. 3.

Gracilaria salicornia: The ^{13}C -NMR spectra of the fractions extracted from *G. salicornia* exhibited the same patterns as shown in Table 1 (the 80%, 40% ethanol, hot water and alkali-treated hot water extracts were not included in the Table), but they differed in remarkable distinct resonance signals

Fig. 6 ^{13}C -NMR spectra of agar fractions from *G. salicornia*

(a) 60% ethanol, (b) autoclave, (c) alkali extracts.

B refers to the carbons in the branched L-galactose.

attributed to 6-*O*-methyl-D-galactose (G') and clear ones to 2-*O*-methyl-L-galactose (A') (Fig. 6, Table 1). The solubility of hydrophobic 6-*O*-methyl-agarose polymer increases with the concentration of ethanol used (15). The relative content of 6-*O*-methyl-agarose in fractions was estimated by using the ratio of the integrals of the carbons of G'6 following the formula $G' = \frac{G'6}{G'6 + G6} \times 100\%$. Here G6 included B6, but the latter was not considered in calculation as it was only in trace amount. 6-*O*-Methyl-agarose (G') was as high as 94.9% in 80% ethanol extract and 88.5% in the autoclave extract (Table 3), implying that the extracts from *G. salicornia* were highly methylated. The intensity of signals of G'4 and G'5 were strong enough to make the signals of G'4 and G'5 disappear (Table 1). All the signals due to the methylated agaroses were alkali-stable (Table 1). In addition, in the NMR spectrum of the autoclave extract the very weak signals assigned to D-galactose 6-sulfate (G⁺), a minor component, were seen as well. In the spectra of most extracts from *G. salicornia* the resonance signal 61.8 ppm was assigned to 4-*O*-methyl, and some weak signals B1-B6 to the branched L-galactose residues (14). In the alkali extract appeared the resonance signals of both 6-*O*-methyl-agarose (G') and floridian starch (C1-C6) (Fig. 6c and Table 1).

$G' = \frac{G'6}{G'6 + G6} \times 100\%$. Here G6 included B6, but the latter was not considered in calculation as it was only in trace amount. 6-*O*-Methyl-agarose (G') was as high as 94.9% in 80% ethanol extract and 88.5% in the autoclave extract (Table 3), implying that the extracts from *G. salicornia* were highly methylated. The intensity of signals of G'4 and G'5 were strong enough to make the signals of G'4 and G'5 disappear (Table 1). All the signals due to the methylated agaroses were alkali-stable (Table 1). In addition, in the NMR spectrum of the autoclave extract the very weak signals assigned to D-galactose 6-sulfate (G⁺), a minor component, were seen as well. In the spectra of most extracts from *G. salicornia* the resonance signal 61.8 ppm was assigned to 4-*O*-methyl, and some weak signals B1-B6 to the branched L-galactose residues (14). In the alkali extract appeared the resonance signals of both 6-*O*-methyl-agarose (G') and floridian starch (C1-C6) (Fig. 6c and Table 1).

Table 4. Relative contents in ratio of absorbances of total sulfate (1250 cm⁻¹), galactose 4-sulfate (850 cm⁻¹), galactose 6-sulfate (820 cm⁻¹) and 3,6-anhydrogalactose (930 cm⁻¹) to total sugar (2920 cm⁻¹) in fractions of *Gracilaria* spp.

Algae	Fraction	Total sulfate					3,6-AG**				
		alkali*	1250/2920	4-sulfate 850/2920	6-sulfate 820/2920	930/2920	1250/2920	4-sulfate 850/2920	6-sulfate 820/2920	930/2920	930/2920
<i>Gracilaria</i> <i>spesstedtii</i>	Cold water		1.52	0.18	0.19	0.05			1.14	1.33	
	60% ethanol		0.13		0.02					1.23	
	40% ethanol		0.48	0.12	0.02	0.02			1.35	1.37	
<i>Gracilaria</i> <i>textorii</i>	Hot water		0.86							0.91	
	60% ethanol		1.12	1.15					0.12	0.16	1.55
	40% ethanol		2.29						0.16		1.81
<i>Gracilaria</i> <i>salicornia</i>	Hot water		4.54	4.05					0.48	0.39	1.01
	60% ethanol		0.63								1.18
	40% ethanol		0.18		0.12				0.10		
<i>Gracilaria</i> <i>salicornia</i>	80% ethanol		0.31		0.14				0.66		
	60% ethanol		0.38		0.07				0.87		
	40% ethanol		0.61	0.38	0.11	0.08			0.60		
Commercial agarose (Marine Colloids product)	Hot water		0.61	0.42	0.14	0.09			0.62	0.72	
	Autoclave		0.61	0.42	0.14	0.09			0.78	0.74	
	Commercial agarose (Marine Colloids product)		0.09		0	0			1.61		

* —: before; +: after alkali modification.

** 3,6-AG: 3,6-anhydrogalactose.

Fig. 7. Infrared spectra of agar fractions from *G. spesstedtii*, *G. textorii* and *G. salicornia*.

A. agarose (Marine Colloids product)

- a. cold water extract from *G. spesstedtii*
- b. cold water extract (alkali-treated) from *G. spesstedtii*
- c. 40% ethanol extract from *G. spesstedtii*
- d. 40% ethanol extract (alkali-treated) from *G. spesstedtii*
- e. 60% ethanol extract from *G. spesstedtii*
- f. 60% ethanol extract (alkali-treated) from *G. spesstedtii*
- g. 40% ethanol extract from *G. textorii*
- h. hot water extract from *G. textorii*
- i. 60% ethanol extract from *G. salicornia*
- j. hot water extract from *G. salicornia*
- k. hot water extract (alkali-treated) from *G. salicornia*
- l. autoclave extract from *G. salicornia*
- m. autoclave extract (alkali-treated) from *G. salicornia*

3. Infrared spectroscopy

The infrared spectra of all fractions including alkali-treated ones from *G. sjostedtii*, *G. textorii* and *G. salicornia* showed the common absorption bands for total sulfate at 1250 cm^{-1} , and 3,6-anhydrogalactose at 930 cm^{-1} typical to the spectrum of agarose (Fig. 7). The absorption bands at 850 cm^{-1} and 820 cm^{-1} were seen individually in the spectra of fractions of these three species of *Gracilaria*. The band at 2920 cm^{-1} due to C-H is a good reference for total sugar content because the number of the C-H group remains constant irrespective of changes in the ratio of galactose to 3,6-anhydrogalactose and sulfate content of the galactans⁽¹⁸⁾. The relative contents of these components in the fractions were estimated by the ratios of the intensity of absorbance at 1250 , 930 , 850 cm^{-1} to that at 2920 cm^{-1} , as shown in Table 4.

The alkali modification of native polymer converted L-galactose 6-sulfate in it to 3,6-anhydro-L-galactose, resulting in a decrease of absorption intensity at 1250 cm^{-1} , an increase at 930 cm^{-1} , and a sharp deepening of the valley at around 800 cm^{-1} (Fig. 7b, d and Table 4).

Gracilaria sjostedtii: In the spectra of fractions from *G. sjostedtii* weak absorbance at 850 cm^{-1} for D-galactose 4-sulfate (C-O-C, ax.)^(1,17) was observed. Its relative content seemed to be higher in cold water extract than in others, but is in general rather lower than that of the total sulfate. In the IR spectra of the cold water and 40% ethanol extracts appeared the absorption band at 820 cm^{-1} , but disappeared after alkali modification, that may be corresponding to L-galactose 6-sulfate (Fig. 7a, b, c, d).

Gracilaria textorii: In all fractions, especially in the hot water fraction, the absorbance ratio for total sulfate ($1250/2920$) was higher than that in the other two species, reflecting the high content of sulfate in the polymer including agarose 6-sulfate, which is well in agreement with the result obtained by ^{13}C -NMR analysis. The absorbance at 820 cm^{-1} attributed to D-galactose 6-sulfate (C-O-S, eq.)⁽¹⁾ was observed to be higher in the hot water fraction as well (Table 4). But the absorbance ratio of $820/2920$ was only about one-tenth that of the total sulfate ($1250/2920$).

Gracilaria salicornia: The absorption band at 850 cm^{-1} for D-galactose 4-sulfate was also observed in the spectra of fractions from *G. salicornia*. Its ratio to 2920 cm^{-1} was higher than that of *G. sjostedtii*. The weak absorbance at 820 cm^{-1} in the autoclave extract showed the presence of a small amount of D-galactose 6-sulfate (Fig. 7l; also see its NMR spectrum, Fig. 6b). An absorption band at 1540 cm^{-1} clearly appeared in the spectrum of the autoclave fraction, indicating the presence of peptide linkage⁽²⁾.

IV. DISCUSSION

The polysaccharides extracted from *G. sjostedtii*, *G. textorii* and *G. salicornia* showed each a different feature in chemical structures. The polysaccharide from *G. sjostedtii* had a high content of hydrophilic D-galactopyranosyl-L-galactopyranose 6-sulfate repeating disaccharides (the precursor to agarobiose), which were more soluble in cold water. Its content amounting to 33.2% of the total agaroses (see above, Table 3) was higher than that in the polymers from most other species of red seaweeds, with the exception of *Porphyra haitanensis*^(1,2), which had the highest (55%) in the polymers of red seaweeds so far as we know. In some species of *Gracilaria* no precursor could be detected in their polymers^(1,11). The difference in content of the precursor may be related to the growth season, the species of algal biosynthesis mechanism, etc. In the polymers from *G. textorii*, all the D-galactose 6-sulfate and 6-O-methyl-D-galactose residues (G) are linked alternately by 3,6-anhydro-L-galactose residues (A) with $\beta(1\rightarrow4)$ and $\alpha(1\rightarrow3)$ linkages, respectively, forming sulfated

and methylated repeating disaccharides in block or at random in the molecules. The cold water fraction of *G. textorii* containing 41% agarose 6-sulfate was not gelable, but the hot water extract having similar content (39%) was weakly gelable after cooling due to its high content of 6-O-methyl-agarose. It has been shown that the polysaccharide extracted from *Gloiopeltis furcata*, an agar-type polymer consisting predominantly of D-galactose 6-sulfate^(1,13), even bearing equimolar 3,6-anhydro-L-galactose residues, had no gelling properties at all. In the infrared spectra of most extracts from *G. textorii*, the absorption band at 820 cm^{-1} , attributed to 6-sulfate on D-galactose, the main component in the polymer, was seen even after alkali treatment of the extracts (Fig. e, f). But the band at 820 cm^{-1} in the spectra of cold water and 40% ethanol extracts from *G. sjostedtii* (Fig. 7a, c) disappeared after alkali modification. This means the band at 820 cm^{-1} was also due to 6-sulfate on L-galactose residues, which will be converted to 3,6-anhydro-L-galactose after alkali treatment. However, the intensity of absorbance at 820 cm^{-1} was not remarkable and related neither to that of the total sulfate at 1250 cm^{-1} nor to the ratio of the integrals of 6-sulfate estimated from the NMR spectra (Table 3) for *G. textorii*, and was too weak for measurement of the peak height for *G. sjostedtii*, so the ratio is not included in Table 4.

The agar polymer of *G. salicornia*, more heterogeneous in structure, was composed of highly methylated agaroses: 6-O-methyl-D-galactose, 2-O-methyl-3,6-anhydro-L-galactose and 4-O-methyl-L-galactose. This agar polymer probably had the highest content of 6-O-methyl-D-galactose (G'), 88.5% in the autoclave extract (the main fraction), and 94.9% in 80% ethanol fraction in comparison with the high contents of 71.3% in the water fraction, 81.4% in the 0.5 mol/L NaCl fraction from *Laurencia flexilis*^(1,12) and 54.8% in the water fraction from *Gelidiella acerosa*^(1,2), estimated by the same formula (see above). The content of 2-O-methyl-agarose was low from the intensity of the signals. Apart from the presence of 6-O-methyl-agarose and 2-O-methyl-agarose there possibly exists 2,6-di-O-methyl-agarose (Fig. 1B(4)). Its chemical shifts are the sum of G' and A': G' 102.7, G' 270.2, G' 382.7, G' 469.0, G' 573.6, G' 671.8, 6-OCH₃ 59.1; A' 198.7, A' 278.9, A' 378.5, A' 497.6, A' 575.7, A' 669.5, 2-OCH₃ 59.2⁽⁶⁾. The methylated agaroses were more hydrophobic, almost insoluble in cold water, and in low yield even in boiling ethanol (Fig. 2C), indicating that they were associated with higher molecular weight in the network and the helical structure of the molecules. Thus, high temperature (autoclave, 121°C) is needed to change the conformation from the insoluble helical agarose molecules to the soluble random coils. In addition, in the fractions from *G. salicornia* there existed some amount of D-galactose 4-sulfate as identified by distinct infrared absorption at 850 cm^{-1} , but it decreased in content after alkali treatment (Table 4). Up to now, the chemical shifts attributed to D-galactose 4-sulfate in the agar polymer in ^{13}C -

Fig. 8 Branched 4-O-methyl-L-galactose attached via C6 to D-galactose residue of agarose

NMR spectra have not yet been assigned, although they have been found early for κ -carrageenan from carrageenophytes [19]. The 4-O-methyl-L-galactoses observed in the ^{13}C -NMR spectra of fractions from *G. salicornia* (Table II) were isolated and verified in the agar from *G. tikvahiae* by Craigie et al. [21]. Lahaye et al. [14] assigned the ^{13}C -NMR signals 61.8 ppm to the carbon of 4-O-methyl and B1-B6 to the branched L-galactose for the agar from the same alga and postulated that the 4-O-galactose was attached to the C4 of the terminal non-reducing L-galactose residue linked with D.

Floridean starch is present in the cells of all red seaweeds as a storage product [6]. In *G. stylioides* it dissolved in hot water and then in alkali extracts, while in *G. textorii* and *G. salicornia* it was only extracted under alkaline condition in the form of amylopectin B with branches. Recently, the signals of floridean starch have been reported in the extract from the marine red alga *Stenogramme interrupta* [7] and in the alkali-treated agar from *Gracilaria eucheumoides* [16].

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