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Chemical composition of agars from a newly reported Japanese agarophyte, *Gracilariopsis lemaneiformis*

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Abstract

The chemical composition of agars from *Gracilariopsis lemaneiformis*, newly reported from Japan, was investigated. Native agars were isolated by a sequential extraction of plants in water at 22 °C and 100 °C, and in boiling 20, 40 and 60% ethanol. Agars in each extract were analyzed by chemical methods, ^{1}H, ^{13}C NMR; and IR spectroscopy. The highest yield of agar (total carbohydrate) was obtained from the 40% ethanol extract (55%). Highest sulfate content was attained in non-alkali treated agars extracted with hot water (4.81%, DS 0.2). The 3,6-anhydrogalactose content was highest in the 40% ethanol extract (36.1% in non-alkali treatment, 40.3% in alkali treatment). The highest methoxyl content (6.51%, DS 0.66) was obtained in the 60% ethanol extract. The *G. lemaneiformis* agar is composed of the biological precursor to agarobiose repeating units and agarobiose containing 6-*O*-methyl agarobiose and a small amount of 2-*O*-methyl-α-L-galactopyranose residues. Alkali treatment improved the chemical quality of the agar fractions, which was comparable with Japanese commercial agar and agarose.

Introduction

Agar from many species of *Gracilaria* and *Gracilariopsis* has been studied for properties and structure by chemical, enzymatic, and spectroscopic methods (Duckworth *et al.*, 1971; Lahaye *et al.*, 1988; Bird & Hinson, 1992; Hurtado-Ponce, 1992). *Gracilariopsis lemaneiformis*, newly reported from Japan, is widely distributed in Tosa Bay, south Ise Bay, and other Japanese inland seas (Chirapart *et al.*, 1994a). The *G. lemaneiformis* was previously referred to as *Gracilaria* sp. (*Chorda* type) by Chirapart & Ohno (1993). The physical properties of its agar vary depending on season (Chirapart & Ohno, 1993), temperature, and growth rate of the seaweed in controlled culture conditions (Chirapart *et al.*, 1994b). However, chemical compositions of agar from this *Gracilariopsis* have not been reported. The purpose of this study was to ascribe the chemical compositions of agar fractions of this new member of Japanese seaweed, based on chemical analysis methods, ^{1}H and ^{13}C NMR, and IR spectroscopy. The agar fractions were obtained by the sequential solvent extraction method (Lahaye *et al.*, 1986).

Materials and methods

Gracilariopsis lemaneiformis was collected from Uranouchi Inlet in Tosa Bay, southern Japan (33° 26′ 35″N, 133° 25′ 00″E) in May 1991. The sample was washed in fresh seawater to remove sand, mud and epiphytes. It was then sun-dried and stored at −30 °C prior to agar extraction. The dried alga was washed overnight in running tap water to remove remaining salt. The sample was cut into small pieces (0.5–1 cm), and left overnight in acetone. It was washed again with fresh acetone until the supernatant solution was clear, and then dried in air.

The agar extraction procedure was modified from the sequential solvent extraction method of Lahaye *et al.* (1986). Seven fractions were then obtained: cold water extract, 100%, 80%, 60%, 40%, 20% ethanol, and hot water extract (100 °C). The cold water extraction was done at room temperature (~ 22–24 °C), by suspending 10 g of dried algal in 1 L distilled water and shaken for 15 h. The supernatant was recovered by filtering with reduced pressure through a glass filter (size no. 17G2). The residues were re-extracted for another 15 h. Both filtrates were analyzed for carbohydrate content, pooled, concentrated *in vacuo*, dialyzed extensively against distilled water, filtered under reduced pressure sequentially through 5, 1.2 and 0.45 μm membrane filters, and freeze-dried. The recovered residues were extracted twice (1.5 h and 1 h) by boiling in 500 mL of 99% ethanol in a 1-L round-bottom flask equipped with a reflux condenser, then subsequently with boiling 80, 60, 40, and 20% ethanol, followed with distilled water at 100 °C for 1.5 and 1 h as mentioned above. The supernatant solution was filtered and treated as for the cold extract. The extracted agars are referred to as the 100%, 80%, 60%, 40%, 20% ethanol, and hot water (100 °C) extract. The agar fractions, except those of the cold water, the 100% and 80% ethanol extracts, were alkali-treated according to Lahaye *et al.* (1986).

Total carbohydrate content was determined for each extract using the phenol-sulfuric acid method (Dubois *et al.*, 1956) using galactose as the standard. 3,6-anhydrogalactose content (3,6-AG) was determined by the resorcinol-acetal method of Yaphe and Arsenault (1965). Sulfate content was determined turbidimetrically with $BaCl_2$ after HCl hydrolysis (Craigie *et al.*, 1984). Methoxyl content was determined by the method of Vieböck and Brecher (1930). The degree of substitution (DS) with sulfate and methoxyl groups was expressed as the number of sulfate and methoxyl groups per disaccharide repeating unit.

^1H and ^{13}C NMR spectra were recorded at 80 °C on JNM-GSX400 spectrometer (Nihon Denshi, Japan) operating at 400 MHz and 100.4 MHz, respectively. The ^1H NMR chemical shifts were measured in ppm and sodium 2,2-dimethyl-2-silapentane-5-sulphonate was used as reference. The ^{13}C NMR chemical shifts are expressed in ppm relative to internal dimethyl sulfoxide (DMSO), and converted to values related to external tetramethylsilane (TMS, conversion constant 39.6).

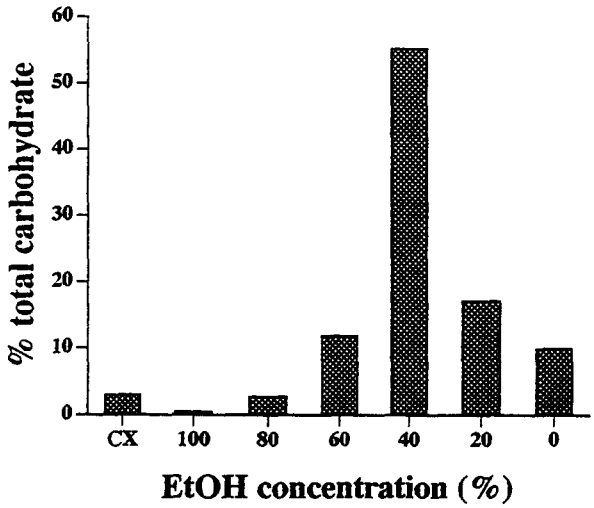

Fig. 1. Yield of agar polymers as percentage of total carbohydrate extracted from *G. lemaneiformis* with cold water at 22 °C (CX), boiling 100, 80, 60, 40, and 20% ethanol solution, and hot water at 100 °C (0).

Infrared spectra of agar films were recorded on a FTIR-4300 spectrophotometer (Shimadzu Co., Kyoto), using KBr as reference.

Results

The yield of each agar polymer extracted was calculated as percentage of total carbohydrate (Fig. 1). Major fractions of agar were recovered with boiling 60, 40, and 20% ethanol. The highest yield was 55%, obtained from the 40% ethanol extract. The lowest yield was 0.46% in the agar extracted with 100% ethanol. Because of the negligible yields from the 100% and 80% ethanol extracts, these fractions were not analyzed further.

3,6-anhydrogalactose (3,6-AG), sulfate, and methoxyl contents, and the degree of substitutions (DS) are shown in Table 1. The 3,6-AG content was higher in both before and after alkali treatments in polymers extracted from the algae with ethanol. After alkali treatment, the 3,6-AG content increased in all the fractions, except that of the hot water extracts that showed no significant increase. The highest 3,6-AG content was obtained from the 40% ethanol extract, before alkali treatment (36.15%) and after alkali treatment (40.33%). High sulfate contents (4.81%, DS 0.20) were observed in non alkali-modified agar extracted with hot water. The content of sulfate decreased in 60% and 40% ethanol extracts, and gradually increased

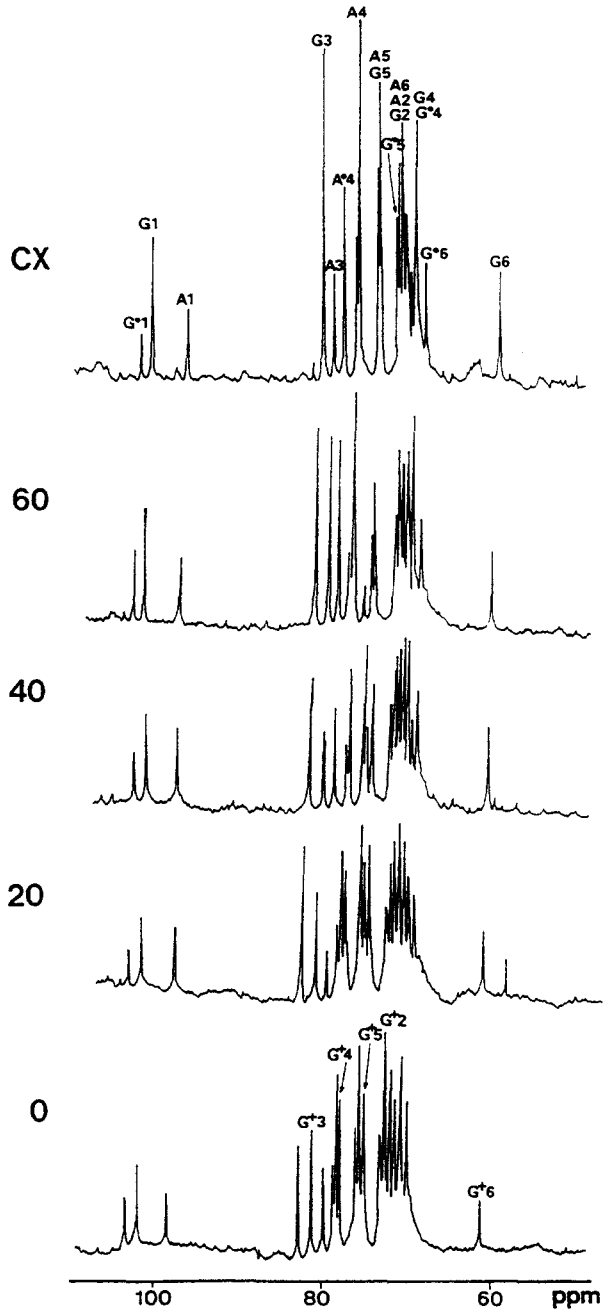

Fig. 2. ^{13}C NMR spectra of non-alkali treated agar polymers extracted with cold water (CX), boiling 60, 40, and 20% ethanol solutions, and hot water at 100 °C (0). G and A refer to carbons in D-galactose and 3,6-anhydro-L-galactose of agarobiose repeating units, respectively; G °, A ° to carbons of D-galactose-6-sulfate and L-galactopyranose and G^+ to carbon of D-galactose-4-sulfate. Chemical shifts were obtained as described in the text.

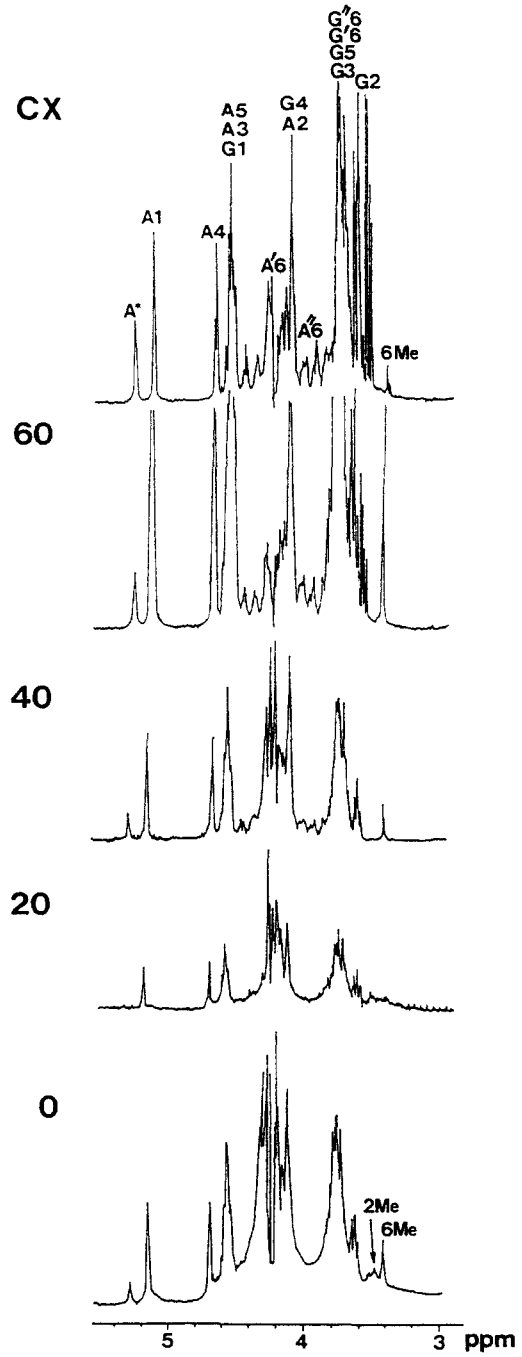

Fig. 3. ^{1}H NMR spectra of non-alkali treated agar polymers. G and A refer to protons of D-galactopyranose and 3,6-anhydro-L-galactopyranose, respectively. G′ and A′ refer to proton of 6-*O*-methyl-D-galactose alternating with 3,6-anhydro-L-galactose. G″ and A″ to proton of D-galactose alternating with 2-*O*-methyl-3,6-anhydro-L-galactose. 6Me and 2Me refer to 6-methylated and 2-methylated agarobiose.

Table 1. Chemical composition of the agar polymers in each fraction extracted from *G. lemaneiformis* collected in Tosa Bay.

Fractions	Content (%)		
	3,6-AG	Sulfate and DS	Methoxyl and DS
Non-alkali treatment			
60% EtOH extract	28.14	3.46 (0.14)	6.51 (0.66)
40% EtOH extract	36.15	3.56 (0.14)	1.66 (0.17)
20% EtOH extract	30.84	4.04 (0.16)	1.55 (0.15)
Hot water extract	23.88	4.81 (0.20)	1.13 (0.11)
Alkali treatment			
60% EtOH extract	33.39	0.63 (0.02)	1.60 (0.16)
40% EtOH extract	40.33	0.52 (0.02)	1.25 (0.12)
20% EtOH extract	34.33	2.65 (0.11)	0.93 (0.09)
Hot water extract	24.03	3.94 (0.16)	0.71 (0.07)

DS degree of substitution per disaccharide repeating unit; data given in parenthesis.

from the 20% ethanol extract to the hot water extract. The increase in methoxyl content was observed in the agar fractions extracted with high concentrations of ethanol. Methylated agar polymer extracted with boiling 60% ethanol before alkali treatment had the highest methoxyl content of 6.51% or DS 0.66. The lowest content was obtained in the hot water extracts (1.13%, DS 0.11). After alkali treatment, the methylated agar polymers were decreased in all the fractions, from 1.6%, DS 0.16 in the 60% ethanol extract to 0.71%, DS 0.07 in the hot water extract.

NMR spectroscopic analysis was conducted on the non-alkali modified agar extracted with cold water (CX), boiling 60, 40, 20% ethanol, and hot water (100 °C). Signal assignment of the agar polymers was achieved by comparison with previously reported chemical shifts (Welti, 1977; Usov *et al.*, 1980; Lahaye *et al.*, 1988; Lahaye & Yaphe, 1989). The ^{13}C NMR analysis (Fig. 2) of these major fractions showed the 12 major signal characteristics of the agarobiose repeat units (G1, 102.0; G2, 70.2; G3, 82.3, G4, 68.6; G5, 75.2; G6, 61.3; A1, 98.2; A2, 69.7; A3, 80.0; A4, 77.2; A5, 75.5; A6, 69.7) corresponding to chemical shifts of model compounds (Usov *et al.*, 1980). The signal at 102.5 (G °1), 68.4 (G °4), 73.0 (G °5), 67.4 (G °6); 77.8 (A °4) attributed to D-galactose-6-sulfate (Lahaye & Yaphe, 1989), was observed in all the agar fractions. The agar fractions of the cold water, 20% ethanol, and hot water extracts had signals attributed to D-galactose-4-sulfate (G^+2:71.0, G^+3:80.0, G^+4:77.0, G^+5:75.0, G^+6:61.3; Lahaye & Yaphe, 1989). The signal attributed to methoxyl groups (59.0 ppm) was not clear, and was observed only in the 20% ethanol extract. However, the ^1H NMR spectra (Fig. 3) showed clearly the signals attributed to methylated agarobiose repeat units. The agar fractions had ^1H NMR spectra confirming qualitatively the degree of substitution with methoxyl obtained by chemical analysis methods (Table 1). All the fractions, except that of the 20% ethanol extracts, showed major signals attributed to proton of 6-*O*-methyl-D-galactose ($\delta = 3.41$ ppm; Lahaye *et al.*, 1988). High intensity of 6-*O*-methyl-D-galactose was obtained from the 60% ethanol extract. Small amount of 2-*O*-methyl-3,6-anhydro-L-galactose-6-sulfate ($\delta = 3.50$ ppm; Welti, 1977) was observed in the hot water extract. An additional signal at 5.28 ppm, referred to H-6 of L-galactose-6-sulfate in the biological precursor to agarobiose (Lahaye *et al.*, 1988), was observed in the agar polymers extracted with cold water, boiling 60 and 40% ethanol, and hot water.

Infrared spectra of the agar fractions extracted both prior to and after alkali modification are shown in Fig. 4A, 4B. The non-alkali and alkali modified agar polymers showed typical IR absorbances of agar at 1250 cm^{-1} for total sulfate esters, 930 cm^{-1} for 3,6-anhydrogalactose, and 891 cm^{-1} for typical sulfate ester (Rochas *et al.*, 1986). In non-alkali treatment, all the agar fractions had absorbances at 820 cm^{-1} and 805 cm^{-1} assigned to the sulfate groups located at C-6 of the D-galactopyranose, and at C-2 of 3,6-anhydrogalactopyranose residues (Rochas *et al.*, 1986), respectively. Absorbance at 850 cm^{-1} attributed to C-4 of D-galactopyranose residues, was observed in the cold water, 20% ethanol, and hot water extracts (Anderson *et al.*, 1968). A small shoulder at 830 cm^{-1} was also observed in all the agar fractions attributed to the sulfate group located at equatorial *O*-2 of D-galactopyranose residues (Anderson *et al.*, 1968). After alkali treatment, the sulfate groups at C-6 were resolved. The region at 805 cm^{-1} disappeared, while at 850 cm^{-1} and 830 cm^{-1}, it was still detectable in the alkali-modified polymers.

Discussion

The properties of agar of *G. lemaneiformis* differ according to season, with the best gel in winter during the period of low algal biomass (Chirapart & Ohno, 1993). The agar extracted from the *G. lemaneiformis*

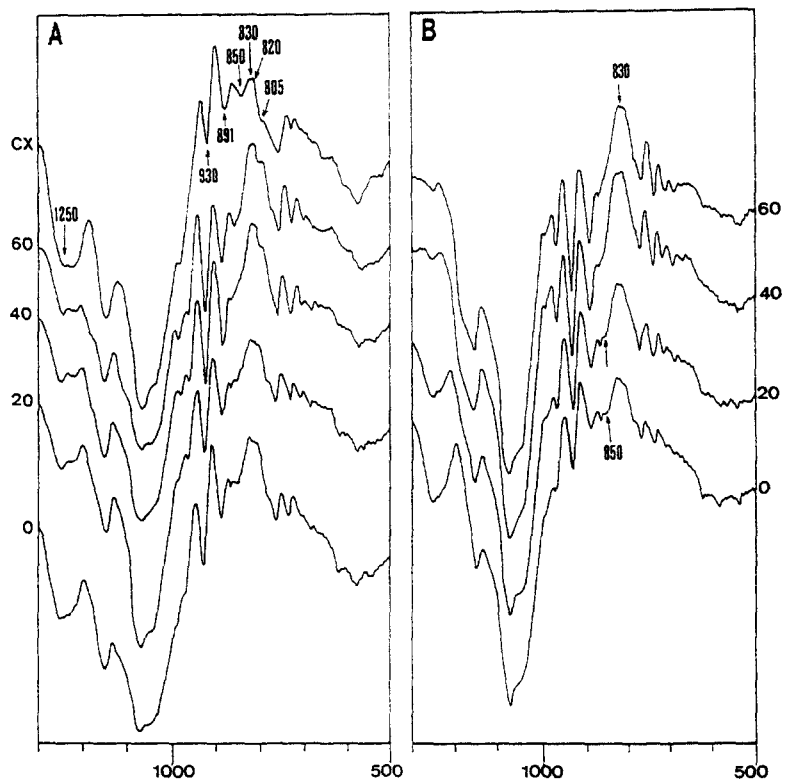

Fig. 4. Infrared spectra of non-alkali (A) and alkali-treated agars (B) extracted with cold water at 22 °C, boiling 60, 40, and 20% ethanol solution, and hot water at 100 °C.

was composed of high 3,6-anhydrogalactose content with sulfate groups substituted at C-6, C-4, and C-2 of agarobiose repeating units. The result was similar to the report on *G. lemaneiformis* from North Carolina extracted in water, which also showed seasonal variation with high gel strength and 3,6-AG content (Bird & Hinson, 1992). Rheological and physical properties of agar from another species of *Gracilariopsis*, *G. heteroclada* from the Philippines, showed a high gel strength after treatment with commercial lime for 3 h (Hurtado-Ponce, 1992). The Japanese agarophyte seems to be a better source of agar compared with previous reports of agars from other species. However, it is difficult to compare these data due to the different analytical methods and environmental factors.

With respect to the present study, the sequential solvent extraction method could isolate the agar polymers enriched with L-galactose-6-sulfate, 6-*O*-methylated agarobiose repeat units and disaccharides repeat units substituted by alkali-stable sulfate groups (Lahaye *et al.*, 1986). Methylated substitution at C-6 of D-galactopyranose and at C-2 of L-galactopyranose has previously been found in *Gracilaria verrucosa* grown in Vietnam (Truong *et al.* 1988), *G. tenuistipitata* and *G. eucheumoides* (Lahaye *et al.*, 1986). The high concentration of methylated agar (6-*O*-methylated agarose) obtained in the 60% ethanol extract was in agreement with previous results (Lahaye *et al.* 1986, 1988) that methylated agars are soluble in high concentrations of hot ethanol-water solutions.

After alkali treatment, the remarkable decrease in the sulfate content (Table 1) can be attributed to the proportion of L-galactose-6-sulfate in the agar fractions extracted with 60, 40, and 20% ethanol that reflected high levels of 3,6-anhydride units. However, the 3,6-AG content in the alkali-treated agar extracted with hot water was not different from the non-alkali treatment. This may be due to the low proportion of the L-galactose-6-sulfate in comparison with other alkali-stable sulfate in this fraction. High sulfate contents in the hot water extract may be caused by sulfation at C-4 and C-2 positions of the galactopyranose residues, as shown by the IR spectra in Fig. 4. According to previous observations, high levels of L-galactose-6-sulfate had been reported in the cold water soluble polymers extracted from young algal tissues of *Gracilaria pseu-*

doverrucosa (Lahaye & Yaphe, 1988). The occurrence of L-galactose-6-sulfate in the cold water, 60% and 40% ethanol, and hot water extracts, corresponded to newly synthesized molecules in young algal tissue that may subsequently synthesize D-galactose-4-sulfate in mature algal tissue (Craigie & Wen, 1984). This sulfation may indicate the proportions of young and mature tissues in the same sample of *G. lemaneiformis*, collected in the same season.

With respect to NMR spectroscopic data in the present study, the expected presence of pyruvated repeating units in the agar fractions was not observed. In contrast, pyruvated agar has been reported in another Japanese seaweed, *Gracilariopsis chorda* (as *Gracilaria chorda* grown in Tosa Bay (Orosco *et al.*, 1992), and in several species of *Gracilaria* (Lahaye & Yaphe, 1989). The signal for methoxyl group corresponding to the C-4 methylation (61.8 ppm) in the agar polymers from *Gracilariopsis lemaneiformis* was not clear. This might be due to the absence of 4-*O*-methyl-L-galactopyranose, or its presence at very low proportions in the non-alkali modified agar. The presence of 4-*O*-methyl-L-galactose in agar has been observed in *Gracilaria tikvahiae* (Craigie *et al.*, 1984), *G. cervicornis* and *G. domingensis* (Lahaye & Yaphe, 1989). Methylation is known to increase gelling temperatures of agars (Guiseley, 1970). There have been reports that the gel strength of agars diminishes markedly with increasing content of 4-*O*-methyl-L-galactopyranose (Craigie *et al.*, 1984). The cause of the methoxyl content decrease of *Gracilariopsis lemaneiformis* agar after alkali treatment is not known. However, it is possibly due to degradation during the treatment.

In conclusion, the agars synthesized from *G. lemaneiformis* were composed essentially of agarobiose repeating units and its biological precursors. The content of 6-*O*-methylated agarobiose repeating units was comparatively high in this agar, and was concentrated in only one highly methylated fraction extracted from the alga with boiling 60% ethanol. Alkali treatment of fractions converted the mainly charged molecules (L-galactose-6-sulfate) to neutral agarose (3,6-anhydrogalactose), particularly that from the 60, 40 and 20% ethanol extracts. The contents of 3,6-anhydrogalactose and sulfate of alkali-treated fractions were comparable to Japanese commercial agar or agarose, and represent an essential commercial source for agar and agarose.

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