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Physical and Chemical Characterization of Agar Polysaccharides Extracted from the Thai and Japanese Species of Gracilaria

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ABSTRACT: Agar polysaccharides extracted from two Thai species of *Gracilaria* (*G. fisheri and G. edulis*) and one Japanese species (*Gracilaria* sp.) were investigated by physical and chemical analysis, and ^{1}H, ^{13}C NMR and FT-IR spectroscopy. Agar with partial 6-*O*-methylated on 3-linked β-D-galactopyranosyl, 2-*O*-methylated on 4-linked 3,6-anhydro-α-L -galactopyranosyl and 4-O-methyl-α-L- galactopyranosyl units attached to the C6 of 3-linked D-galactopyranosyl units were isolated from *G. fisheri.* The large parts of 6-*O*-methylated on 3-linked β-D-galactopyranosyl units and partial methylation on C2 of 4-linked 3,6-anhydro-α-L -galactopyranosyl units were observed in the agar extracted from *G. edulis* which corresponded with higher gelling temperature (»60 C°). In contrast, the Japanese agar extracted from *Gracilaria* sp. showed a typical pattern of agarobiose with partial methylation at C6 of the D-galactopyranosyl units. All agars extracted exhibited sulfate substitution at different positions in the polymers. High sulfate contents were obtained in native agar of *G. fisheri* (4.56%) and *G. edulis* (7.54%) that mainly branched at C-4 of the D-galactopyranosyl unit. The presence of this unit was responsible for poor gelling ability of the agar polymers. Alkali treatment was effective both in removing alkali-labile sulfate and increasing the gel strength in *Gracilaria* sp. (334.5 ± 14.1 g/cm^2) whereas only a slight effect was noted on *G. fisheri* (228.27 ± 48.18 g/cm^2) and *G. edulis* (239.95 ± 28.35 g/cm^2). Further investigation may need to determine the constituent sugars and an alternative utilization of the Thai gracilaroids.

KEYWORDS: *Gracilaria fisheri, Gracilaria edulis, Gracilaria* sp., Agar, Galactan.

INTRODUCTION

Agars are known as water-soluble, gel-forming polysaccharide extracts from agarophyte members of the Rhodophyta. Agars are usually composed of repeating agarobiose units alternating between 3-linked β- D-galactopyranosyl (G) and 4-linked 3,6-anhydro-α- L- galactopyranosyl (LA) units. This disaccharide regularity may be marked or modified in a number of ways by substitution of hydroxyl groups with sulfate hemiesters and methyl ethers in various combination and more rarely with a cyclic pyruvate ketal as 4,6-O-[(R)-1-carboxyethylidene] acetal and sometimes by additional monosaccharides[1] . Typical constituents found in agar group polysaccharides are shown on Figure 1. Moreover, the pattern of substitution groups depends on the species, various environmental and physiological factors, and the procedures used in extraction and isolating agar[2,3]. The yield and physical properties of agar such as gel strength, gelling and melting temperature as well as chemical properties, determine its value to the industry.

Members of the genus *Gracilaria* are widely distributed geographically with the major species being reported from warm-water and tropical regions, with

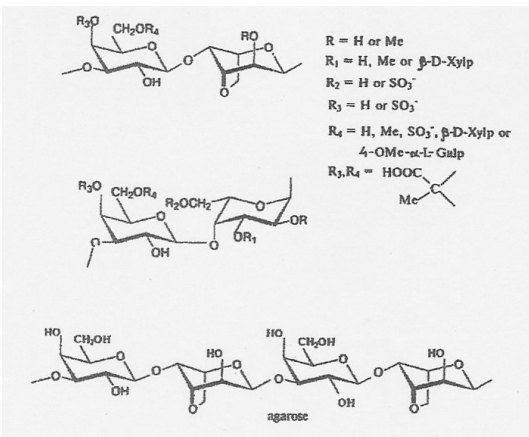

Fig 1. Structural features of the agar group of polysaccharides[1].

18 species reported in Thailand [4-7]. *G. fisheri* and *G. edulis* are common marine algae in Southern and Eastern Thailand, respectively. *G. fisheri* is a well known source of agar and has been exploited and processed locally while *G. edulis* has been used in many parts of the world[8]. However, the chemical structure of agar from Thailand species of *Gracilaria* has not been reported.

The present study is to investigate the physicochemical properties and chemical structure of the native and alkali treated agar extracted from Thai species of *Gracilaria* (*G. fisheri, G. edulis*) and Japanese species (*Gracilaria* sp.)

Materials and Methods

Samples of *Gracilaria fisheri* (Xia *et* Abbott) Abbott and *G. edulis* (Gmelin) Silva were gathered in March 2004 from Samut Songkhram Research Station, Faculty of Fisheries, Kasetsart University, and during December 2003 to March 2004 from Ban Ang-Sila, Chon Buri province, in the east of Thailand, respectively. Another sample of *Gracilaria* species was collected in July 2004 from the Shimonoseki Sea, Yamaguchi Prefecture, Japan. The samples were washed in fresh water to remove sand, mud and epiphytes, and then air-dried. The dried samples were washed overnight in running tap water to remove remaining salt. The samples were cut into small pieces (0.5-1 cm) and left overnight in acetone to remove colors and then washed again with fresh acetone until the supernatant was colorless. The cleansed seaweeds were air dried prior to agar extraction.

Agar Extraction

Two experiments of total agar extraction with and without NaOH treatment were carried out. The dried sample of 30 g was boiled for 2 hours with 900 ml of distilled water and used for non-alkali treatment (native agar). Another 30 g sample was incubated in 2 liter of 5% NaOH solution at 80 °C for 2 hours. The algae were washed in running tap water for 30 min to remove excess NaOH. The alkali-treated algae were neutralized in 2% H_2SO_4 solution for 1 hour, then washed in running tap water overnight until complete elimination of the acid. The agars were extracted by boiling water as described above. The extracted agar solution was filtered through a muslin bag while hot and the extracted agar was kept at room temperature until it solidified. The solidified agar was cut into strips, then frozen at -20°C for 48 hours, and thawed in tap water to the hydrogel and dried.

Physical Properties

Gel strengths in g cm^{-2} were determined at 20°C for 1.5 % agar solution with a Nikansui gel tester, in according with the methods described[9]. The gel strength meter has a 1.0 cm^{-2} plunger to which weight was added sequentially, beginning with 100 g, until the plunger broke the gel surface. The time between the addition of the last weight and the point where the plunger reached the bottom of the beaker was determined using three replicates, for each sample. The gel strength will be expressed as the g cm^{-2} that the gel resisted for 20 sec. Under this condition, commercial agar (Pearl Mermaid, Thailand) and agarose (HIMEDIA®) were used as references and their gel strengths were 189.09 ± 28.12 and 709.38 ± 43.18 g cm^{-2}, respectively. Viscosity determination was conducted at 80 °C using Brookfield Synchrolectric Viscometer (Model DV-III). Gelling temperature was determined by dissolving 1.5% (w/v) of agar in distilled water and pouring 10 ml of agar solution into a 18 x 150 mm test tube with a thermometer placed inside the tube. The tubes were immediately placed into a rack in a 60 °C water bath. When the temperature in the tubes was the same as the bath, cold water was passed through a copper coil in the bath. When the temperature in the tubes reached 50°C, the flow rate of the cold water was adjusted so that temperature dropped about 0.3-0.5 °C per minute and then the thermometers were withdrawn from the tubes. When gelling occurred the temperature was recorded as the gelling temperature[10]. To determine the melting temperature, the technicians prepared 1.5% (w/v) of agar solution by pouring 5 ml of the agar solution into 10 x 100 mm screw cap tubes, and allowing the solution to gel overnight at room temperature (25 °C). Next the tube was placed upside down in a rack in 60°C water bath. Technicians gradually raised the temperature of the bath at 1 °C per minute. When the gel melted and fell to the bottom of the tube the temperature was recorded as the melting temperature.

Chemical Properties

The 3,6-anhydrogalactopyranosyl content (3,6-AG) of agar extracted was determined by the resorcinol-acetal technique using fructose as a reference[11]. Sulfate content of extracted agar was measured turbidimetrically with $BaCl_2$ after HCl hydrolysis[12]. All physical and chemical properties were performed in three replicates.

Spectroscopy

^{13}C and ^{1}H NMR spectra of 4-5% (w/v) of agar solutions in D_2O were recorded at 90°C on a Bruker AVANCE 400 spectrometer equipped with a 5 mm probe at a base frequency of 100.62 MHz and 400.13 MHz, respectively. Chemical shifts were reported relative to an internal acetone standard at 31.26 ppm (^{13}C NMR) and 2.75 (^{1}H NMR). For ^{13}C NMR spectroscopy, the

pulse sequence was with *D1* = 2.00 and *AQ* = 1.30 s. For ^{1}H NMR spectra all samples were submitted to a delay (*D1*) and acquisition time (*AQ*) of 1.00 s and 3.96 s, respectively. The methoxyl content was determined by ^{1}H NMR spectroscopy, using presaturation of the residual HOD signal[13]. The Fourier-transform (FT-IR) spectra were recorded on Nicolet Impact 410 FT-IR spectrometer. The samples were analyzed as KBr pellet. Baselines of spectra were corrected in the 2,500-650 cm^{-1}.

Results

The agar yields, and the physical and chemical properties, of the three species are shown in Table 1. The alkali-treated agar from *G. fisheri* was obtained in a remarkably high yield (39.55% w/w), but agar from this species consistently exhibited low gel strength. The agar yields of *G. edulis* were 10.90 and 34.34 % (w/w) in native and alkali treated material, both with low gel strength. The highest gel strength was found for the agar extracts of *Gracilaria* sp. The alkali treatment improved the gel strength from 202.31 g cm^{-2} in native agar to 334.50 g cm^{-2}. Agar yields of this species were 39.42and 31.30 % (w/w) in native and alkali treated, respectively. Among the species investigated, the highest gelling (60-61 °C) and melting (87-92 °C) temperature were found on agar from *G. edulis*. The gelling and melting temperatures of the agars from *G. fisheri* were lower than those of the other species.

Chemical analysis showed an inverse relationship between sulfate and 3,6-anhydrogalactose content. The alkali treated agar had lower sulfate content than native agar (Table 1). The native agar from *G. edulis* had the greatest sulfate content (7.54 % w/w); the species with lowest sulfate content and with the highest 3,6-anhydrogalactose content after alkali treatment was *Gracilaria* sp.

NMR and FT-IR Spectroscopy

The native agars obtained from *G. fisheri* and *G. edulis* had a very dark brown color while native agar

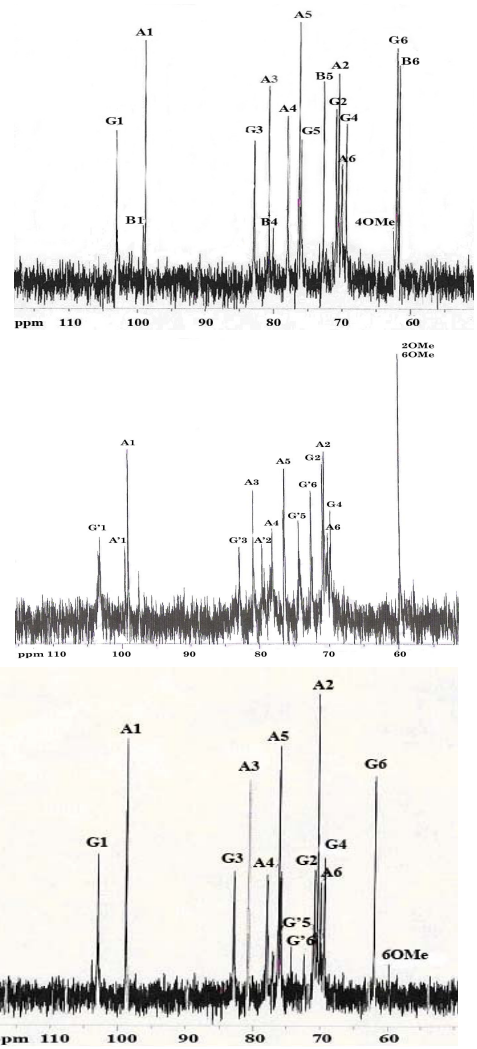

Fig 2. ^{13}C NMR spectra of alkali-treated agars from *G. fisheri* (a), *G. edulis* (b) and *Gracilaria* sp. (c). G and A refer to carbon in D-galactopyranosyl and 3,6-anhydro-L-galactopyranosyl of agarobiose, respectively. G' and A' refer to carbon in 6-*O*-methyl-D-galactopyranosyl and 2-*O*-methyl-3,6-anhydro-L-galactopyranosyl, respectively.B refers to 4-*O*-methyl-L-galactopyranosyl; OMe refers to the carbon of the methyl group.

Table 1. Yield and quality of native and alkali-treated agars from *G. fisheri, G. edulis* and *Gracilaria* sp. (Mean ± SE, n = 3).

	G. fisheri		*G. edulis*		*Gracilaria* sp.	
	native	alkali-treated	native	alkali-treated	native	alkali-treated
Yield (% w/w)	13.33 ± 1.78	39.55 ± 7.59	10.90 ± 0.92	34.34 ± 1.74	39.42 ± 0.71	31.30 ± 1.79
Gel strength (g/cm^2)	145.61 ± 34.55	228.27 ± 48.18	197.08 ± 72.87	239.95 ± 28.35	202.31 ± 7.39	334.50 ± 14.1
Viscosity (cP)	18.37 ± 0.55	4.34 ± 0.27	22.05 ± 2.76	8.59 ± 0.37	57.33 ± 3.51	5.28 ± 0.23
Gelling temp.(°C)	49.25 ± 0.96	47.00 ± 0.00	60.20 ± 0.45	61.00 ± 1.00	53.40 ± 0.55	53.6 ± 0.89
Melting temp.(°C)	72.40 ± 0.10	72.37 ± 0.06	92.60 ± 0.30	87.63 ± 0.06	82. 00 ± 0.00	75.00 ± 0.00
Sulfate content (% w/w)	4.56 ± 0.14	2.80 ± 0.04	7.54 ± 0.18	4.92 ± 0.07	3.75 ± 0.06	0.40 ± 0.04
3,6-AG content (% w/w)	35.19 ± 0	38.88 ± 1.88	37.03 ± 2.83	43.06 ± 0.41	38.11 ± 2.84	52.55 ± 7.24

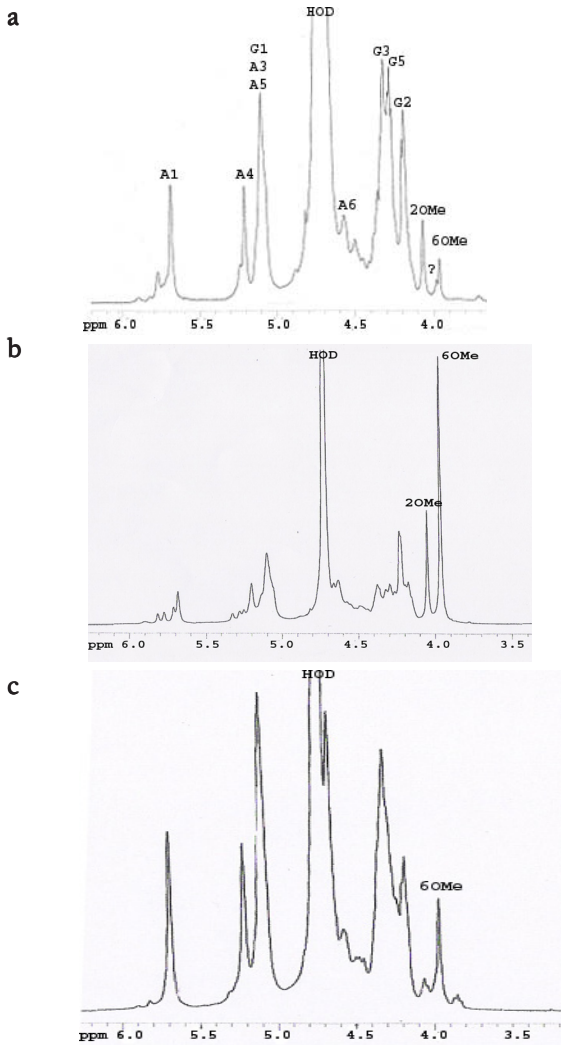

Fig 3. ^{1}H NMR spectra of alkali-treated agars from *G. fisheri* (a), *G. edulis* (b) and *Gracilaria* sp. (c). G and A refer to carbon in D-galactopyranosyl and 3,6-anhydro-L-galactopyranosyl of agarobiose, respectively ; OMe refers to the hydrogen of the methyl group.

obtained from *Gracilaria* sp. had a yellow-green color. The darker color resulted in a lower resolution of NMR and in difficulty identifying the anomeric protons and the coupling constants. The ^{13}C NMR spectra of all alkali-treated samples investigated are shown in 12 signals assigned to the carbon of agarobiose units[14-16] as shown in Figure 2. It should be noted our chemical shifts were approximately 1 ppm downfield to the value generally quoted in the literature due to our use of internal acetone at 31.62 ppm. The signals at 103.03, 70.86, 82.83, 69.36, 75.96 and 62.03 ppm corresponded to the 3-linked β-D-galactopyranosyl units, while the signals at 98.88, 70.50, 80.73, 77.96, 76.25 and 70.02 ppm corresponded to the 4-linked 3,6-anhydro-α-L-galactopyranosyl units. Moreover, additional signals in the spectra revealed the presence of substituted agarobiose repeating units. The ^{1}H NMR signals of all the alkali-treated samples were assigned by comparing the chemical shifts observed from *G. crassissima*[13] and those reported for 2-O-methylation on 4-linked 3,6-anhydro-a- L-galactopyranosyl units (2OMe) and for 6-O-methylation on 3-linked β-D-galactopyranosyl units (6OMe) from *G. dura*[17]. As well as ^{13}C NMR, it should be noted our chemical shifts were approximately 0.5 ppm downfield to the value generally quoted in the literature due to our use of internal acetone at 2.75 ppm.

^{13}C NMR spectroscopic analysis of alkali treated agar from *G. fisheri* is shown in Figure 2a. The additional signals (B1: 99.14, B4: 80.80, B5: 72.71 and B6: 61.69) are assigned to the proposed 4-*O*-methyl-α-L-galactopyranosyl, which is present as a branch in this agar by attachment to the C6 of some of the 3-linked β-D-galactopyranosyl units[13,18]. The chemical shifts for the 6OMe and 2OMe at ~60 ppm were not observed. In contrast, the signals at 3.96 ppm and 4.07 ppm in the ^{1}H NMR spectra (Figure 3a) were assigned to the 6OMe and 2OMe, which were observed with a degree of methylation (DS) per disaccharide of 0.33 and 0.17, respectively. These results indicated little 6-*O*-methylation and 2-*O*-methylation in *G. fisheri* agar. Furthermore, there is no evidence for 4OMe in ^{1}H NMR spectra. The possibility was that the 4OMe merged with 6OMe or 2OMe signal due to the unknown signal observed at 3.98 ppm. The FT-IR spectra (Figure 4) showed a strong absorbance at 930 cm^{-1} attributed to vibration of the C-O-C bridge of 3,6-anhydrogalactose[19]. The small signals at 850 and 868 cm^{-1} were attributed to D-galactose-4-sulfate (G4S) and the shoulder of L-

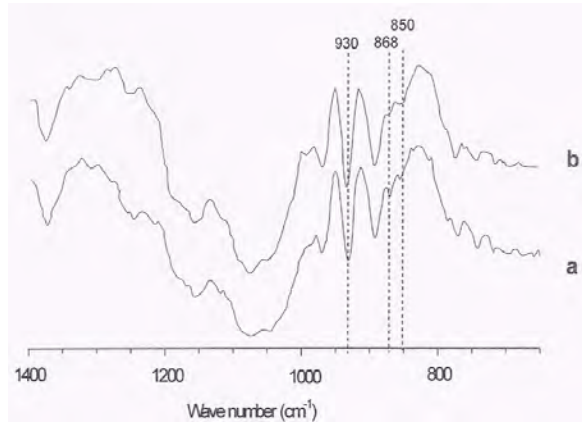

Fig 4. FT-IR spectra of *Gracilaria fisheri*. (a) native agar and (b) alkali-treated agar. Peaks are attributable to 3,6-anhydrogalactose (930 cm^{-1}), L-galactose-6-sulfate shoulder (868 cm^{-1}) and D-galactose-4-sulfate (850 cm^{-1}).

galactose-6-sulfate of agar polymer[20], respectively.

The alkali treated agar from *G. edulis* had a very highly methylated agarose structure. The ^{13}C-NMR spectra (Figure 2b) of agar extracts exhibit similar signals, which were attributed to an agarose with methylation occurring at C6 of β-D-galactopyranosyl and C2 of 3,6 anhydro-α-L-galactopyranosyl units. The spectrum contained the major coalescing signal of the two-methoxyl carbon which appears as one peak at 59.96 ppm[13]. Complete methylation was apparent at C6 positions of D-galactopyranosyl units by the absence of C6 non-methylated D-galactopyranosyl signal at 62.5 ppm (G6). This indicated that the agar isolated from *G. edulis* is a highly methylated polysaccharide. These results corresponded with high gelling temperature (» 60 °C) of agars in Table 1. The ^{1}H-NMR (Figure 3b) showed strong signals at 3.96 ppm corresponding to 6OMe with DS as 1 and minor signal of 2OMe (4.07 ppm) with DS was 0.42. The FT-IR spectra (Figure 5) indicates the presence of sulfation on C4 of β-D-galactopyranosyl units (G4S) at 855 cm^{-1}. Moreover, the additional spectra at 827 cm^{-1} attributed to D-galactose-2-sulfate (G2S)[20] were detected in both native and alkali treated agar.

The alkali-treated agar from *Gracilaria* sp. had a partially methylated agarose structure. Its ^{13}C-NMR spectra (Figure 2c) contained weak signal for 6OMe at 60.00 ppm indicating partial methylation at C6 of the 3-linked α-D-galactopyranosyl units which was corroborated by resolution of weak signals at 74.35 and 72.41 ppm attribute to C'5 and C'6 of 6-*O*-methyl-α-D-galactopyranosyl units (G6M). A methyl proton signal was resolved in the ^{1}H NMR spectra at 3.95 ppm indicated 6OMe with DS as 0.08 (Figure 3c).

The presence of sulfate ester obtained by infrared

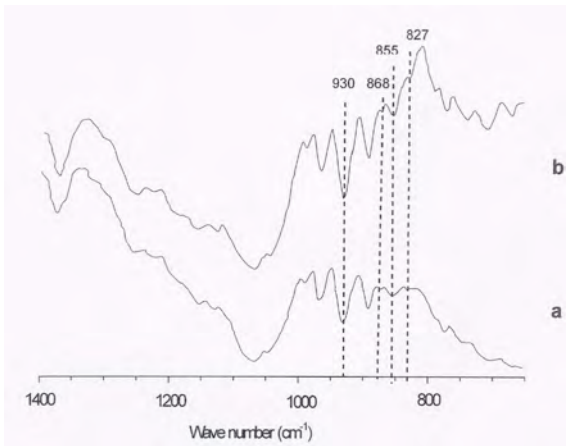

Fig 5. FT-IR spectra of *Gracilaria edulis*. (a) native agar and (b) alkali-treated agar. Peaks are attributable to 3,6-anhydrogalactose (930 cm^{-1}), L-galactose-6-sulfate shoulder (868 cm^{-1}), D-galactose -4-sulfate (855 cm^{-1}), and D-galactose-2-sulfate (827 cm^{-1}).

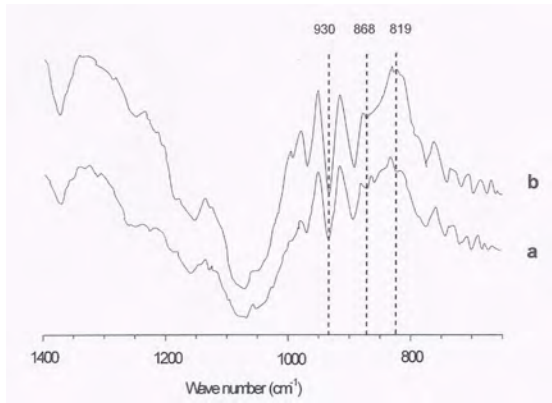

Fig 6. FT-IR spectra of *Gracilaria* sp. (a) native agar and (b) alkali-treated agar. Peaks are attributable to 3,6-anhydrogalactose (930 cm^{-1}), L-galactose-6-sulfate shoulder (868 cm^{-1}), and D-galactose -6-sulfate (819 cm^{-1}).

spectrum (Fig. 6) indicated a C-O ether bond of 3,6-anhydro-L-galactose at 930 cm^{-1} and the small signal at 819 cm^{-1} was assigned to D-galactose-6-sulfate[19,20] which still persisted after alkali treatment (Figure 6b). This indicated the presence of alkali-stable-6-sulfate on 3-linked α-D-galactopyranosyl units.

There was no evidence for sulfation in the ^{13}C NMR spectrum of all alkali treated samples, indicating that this substitution may be below the limit of detection. Furthermore, the signal assigned to disaccharides containing L-galactose-6-sulfate (LA6S) at 68.7 ppm disappeared in all alkali-treated samples. Signals were not detected for agar substituted with pyruvate ketal using proton NMR in any sample.

Discussion

The low yield extraction of native agar from *G. fisheri* and *G. edulis* (Table 1) may be due to a high content of non-gelling water soluble agar and leaching out during the extraction process[21]. In the case of *Gracilaria* sp., the decrease of agar yield after alkali treatment appears to be related to degradation of polysaccharides during treatment and agar loss by diffusion during processing. This result is in agreement with previous reports regarding the yield loss due to alkaline degradation[21].

Chemical analyses showed inverse relationship between sulfate content and 3,6-anhydrogalactopyranosyl, while the alkali treated agar had lower sulfate content than native agar (Table 1). According to Rees[22], sulfate units at points in the polysaccharide chains comprising the gel cause kinks in the helical structure responsible for gel formation. This results in agars of lower gel strength. It can be improved by alkali treatment of agar molecules

eliminating the unstable axial sulfate groups at C6 of L-galactopyranosyl units and converts it into the anhydride units, giving rise to a more stable 3,6-anhydrogalactose[23]. Therefore, the presence of alkali stable G4S and G2S were responsible for poor gelling ability of agar from *G. fisheri* and *G. edulis*. The species with lowest agar sulfate content was *Gracilaria* sp.

The unusually high gelling temperature (»60 °C) in *G. edulis* agar has been positively correlated to natural methoxyl content[10]. A similar result was obtained from *G. arcuata* collected from the Philippines[21]. The decrease in the melting point after alkali treatment may be caused by alkali hydrolysis reducing its molecular weight (Table 1). These results can be confirmed by viscosity measurements. The viscosity of agar solution at constant temperature and concentration is a direct function of the average molecular weight[24].

The methyl content of an agar of *Gracilaria* species origin was mainly attributed to 6-*O*-methylated-D-galactopyranosyl and 2-*O*-methylated-L-galactopyranosyl units[24]. The alkali-treated agar from *G. fisheri* had partially methylated agarose structure. Its NMR spectrum (Figure 2a and 3a) contains minor resonance characteristics of 2-*O*-methylated-3,6-anhydro-α-L-galactopyranosyl, 6-*O*-methylated-β-D-galactopyranosyl, 4-*O*-methyl-β-L-galactopyranosyl attached on C-6 of 3-linked -β-D-galactopyranosyl units and presence of sulfation on C4 of D-galactopyranosyl units. The presence of 4-*O*-methyl-L-galactopyranosyl in agar also has been observed in *G. crassissima*[13], *G. varicosa*[18] and *G. tikvahiae*[25]. This sugar unit may be attached as a side group and the gel strength of agars diminishes markedly with increasing content of 4-*O*-methyl-L-galactopyranosyl and its content depends on the tissue age[25].

The alkali-treated agar extracted from *G. edulis* is composed entirely of 6-O-methylated at the 3-linked β-D-galactopyranosyl units, and partially 2-*O*-methylated at 4-linked 3,6-anhydro-α-L-galactopyranosyl units (G6M-LA + G6M-LA2M structure) and sulfation is present on C4 and C2 of D-galactopyranosyl units. The methylation on both C6 of D-galactopyranosyl and C2 of D-galactopyranosyl units had been observed in agar from *Curdiea* species[15] and *G. dura*[17]. A similar result was obtained from *G. edulis* collected from the Philippines[8]. However, there were clear differences from *G. edulis* collected from Fiji with unmethylated but 13% of 3-linked-β-D-galactopyranosyl units bearing 4,6-*O*-pyruvate acetal substituents[16].

The agar extracted from *Gracilaria* sp. is composed of partial 6-*O*-methylate-β-D-galactopyranosyl with DS as 0.08 (G-LA + G6M-LA structure) and has sulfation on C6 of D-galactopyranosyl units. Similarly, the simultaneous occurrence of methylation and sulfation at C6 of the β-D-galactopyranosyl units has been reported from *G. multipartite*[24] and *G. cervicornis*[26].

In conclusion, methylation can occur extensively at C6 of D-galactopyranosyl units, C2 of 3,6-anhydro-α-L-galactopyranosyl units or at both positions. Unusual 4-*O*-methyl-α-L-galactopyranosyl attached on C6 of D-galactopyranosyl units was found in agar from *G. fisheri*. The amount and position of methylation can affect the gel-forming mechanism of agar that allows gel to form at higher temperatures in *G. edulis* agar. The agar extracted from *Gracilaira* sp. might be considered for exploitation as a source of food grade agar since its alkali-treated agars were obtained in acceptable yield and gel strength and gave gels with good clarity and low color. Further work is needed to determine the constituent sugar(s) in the three agar samples by GLC and to find an alternative utilization of agar extracted from *G. fisheri* and *G. edulis*.

Acknowledgements

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