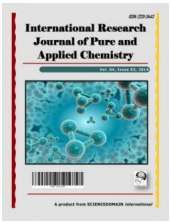

International Research Journal of Pure & Applied Chemistry

17(2): 1-11, 2018; Article no.IRJPAC.45201
ISSN: 2231-3443, NLM ID: 101647669

Discovery of κ-Carrageenan-Like Agarose from a Red Seaweed, *Gracilaria coronopifolia*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IRJPAC/2018/45201
Editor(s):
(1) Dr. Bengi Uslu, Department of Analytical Chemistry, Ankara University, Ankara, Turkey.
Reviewers:
(1) Bilal Muhammad Khan, Shantou University, PR China.
(2) Hidetaka Kawakita, Saga University, Japan.
Complete Peer review History: http://www.sciencedomain.org/review-history/27934

Original Research Article

Received 23 September 2018
Accepted 07 December 2018
Published 22 December 2018

ABSTRACT

Agarose is a major gelling constituent of agar extracted from red seaweeds and is used in the separation, purification, and characterization of bio-macromolecules (DNA, RNA, protein and polysaccharide). The purpose of this study is to identify a novel agarose molecule. The polysaccharide was isolated from a red seaweed, *Gracilaria coronopifolia* which is grown in Okinawa, Japan. The yield of the polysaccharide was 15.2% (W/W), and the total carbohydrate and sulfuric acid were 69.5% and 20.3%. β-D-Galactoside and 3,6-anhydro-α-D or L-galactoside were identified from the methanolyzate of the polysaccharide by thin-layer chromatography. Galactose-4-sulfate and anhydro-galactose were identified by Fourier Transform Infrared (FTIR) spectrum of the polysaccharide. The specific rotation $[\alpha]_{589}$ was -2.0°. From the ^{13}C- and ^{1}H-NMR spectra, 1,3-linked β-D-galactose-4-sulfate and 1,4-linked 3,6-anhydro-α-D- or L-galactose were assigned. The polysaccharide did not gel even at 0.5% (W/W) at room temperature, but very weak and transparent gel was observed in 0.2% solution with addition of 13.5 mM KCl. We concluded the polysaccharide isolated from *G. coronopifolia* was the κ-carrageenan-like agarose, the structure of which was proposed. This work is the first to report on the agarose involving 1,3-linked β-D-galactose-4-sulfate.

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Keywords: Gracilaria coronipifolina; ^{1}H and ^{13}C-NMR analysis; κ-carrageenan-like agarose; transparent gel; chemical structure.

1. INTRODUCTION

Agarose and carrageenans are water-extractable galactan from red algae and used in food, cosmetic, chemical analysis and biotechnology as gelling agents. They are composed of repeating disaccharide units of β-(1→3)-D-galactose and α-(1→4)-3,6-anhydro-L-galactose residues for the agarose and sulfate groups substituting at C-4 position of β-D-galactose for κ-carrageenan, and sulfate groups substituting at C-4 position of β-D-galactose and C-2 position of 3,6-anhydro-α-D-galactose residues for ι-carrageenan. One of the authors, Tako, isolated agar (agarose) [1] and methylated agar (agarose) [2] from red seaweeds grown in Okinawa Islands, Japan. Tako also reported κ-carrageenan [3], ι-carrageenan [4], and pyruvated ι-and λ- hybrid carrageenan [5] from subtropical biomasses.

In the course of rheological study, Tako proposed gelation mechanism for κ-carrageenan [6-8], ι-carrageenan [9,10] and agarose (agar) [11]. As shown in Fig. 1, an intramolecular K^{+}-bridge on κ-carrageenan molecule between sulfate oxygen of C-4, which oriented at axial configuration of D-galactosyl residue, and 3,6-ring oxygen of anhydro-α-D-galactosyl residue [6]. The bridge built up at low temperature, owing to the decrease in kinetic energy and Brownian motion of the polymer molecules and water. The intramolecular bridge, which was preferentially placed to make polysaccharide molecule rigid, was the first demonstration of this effect at a molecular level. On the basis of the intramolecular bridge, an intermolecular association of the κ-carrageenan molecules was developed. As the kinetic energy and Brownian motion of the polysaccharide molecules and water decrease at low temperature, many intramolecular cation-bridges serve to keep rigid the chains, which result in intermolecular association. Consequently, we proposed an intermolecular association between sulfuric acid groups on different molecules with electrostatic forces of attraction [7,8], as shown in Fig. 1.

Tako also proposed that the ι-carrageenan (Ca salt) involves an intramolecular association through Ca cations with ionic bonding between sulfate oxygens, as illustrated in Fig. 2 [9,10]. Each cation is co-ordinated to two sulfate groups by ionic bonding. Since ionic bonding is stronger

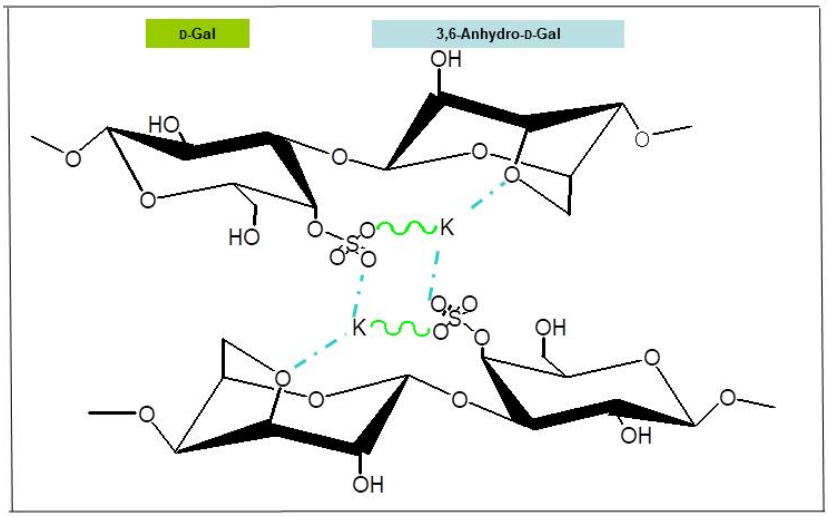

Figure 1. Intra- and intermolecular associations of κ-carrageenan molecules in aqueous solution.

Associations: (∿∿) Ionic bonding; (– . –) electrostatic forces of attraction. (Reproduced with permission from references 14 and 15. Copyright 2014 and 2015 Scientific Research Publishing).

than electrostatic forces of attraction, the molecular chain keep rigid even at room temperature and, therefore, intermolecular associations take place. Thus an intermolecular Ca-bridges take place on different molecules having electrostatic forces of attraction (Fig. 2). The intra- and inter-molecular cation bridges of the Ca-salt of ι-carrageenan differs essentially from that of the K-salt of κ-carrageenan, because the former consists of double ionic forces and electrostatic forces of attractions, whereas the latter consists of single ionic force and triple electrostatic forces of attraction. These different mechanisms provide an explanation for the conformational transition of ι- and κ-carrageenan in aqueous solution. Since ionic forces are stronger than electrostatic forces of attraction, the molecular chains become rigid even at intermediate temperature (45°C) which was conformational transition temperature. Intra- and inter-molecular Ca-bridges dissociate above the transition temperature.

Tako, furthermore, proposed gelation mechanism of agarose as shown in Fig. 3 [11]. An intramolecular hydrogen bonding between OH-4 of the β-D-galactose and the adjacent hemiacetal oxygen atom of the anhydro-α-L-galactose residue. Intermolecular hydrogen bonding was also proposed between the ring *O*-3,6-atom and the OH-2 which oriented at axial configuration of anhydro-L-galactose residues on different molecules (Fig. 3). The intermolecular hydrogen bonding results from the cage effect of the anhydro-α-L-galactose residues which adopt a tetrahedral distribution and therefore attract not only each other, but also water molecules with hydrogen bonding. The gelation mechanism (intra- and intermolecular hydrogen bonding) of agarose molecules has been supported by ^{1}H- and ^{13}C-NMR spectroscopy [12].

The gelation mechanisms proposed led to road on the discussion the role of agarose molecules including water in principle [13-15]. The association resulted in cage effect, which led to the lowest energy state of electrons of the lone pairs of ring oxygen atoms of the anhydro-L-galactopuranosyl residues and water molecules. The conformation adopted a tetrahedral distribution, and therefore, attracted not only each other but also water molecules due to hydrogen bonding. The intra- and inter-molecular hydrogen bonding of agarose molecules resulted in gelation up to a high temperature (<60 °C), and their periphery was surrounded by hydrophobic carbon and hydrogen atoms, which were required to play a role in the hydrophobic effect. The hydrophobic and cage effects led to hydrogen bonding that easily occurred within water molecules because of a decrease in entropy. Therefore, hydrogen bonding also took place easily with tetrahedral water molecules on the outside of the polymer helices (Fig. 4). The

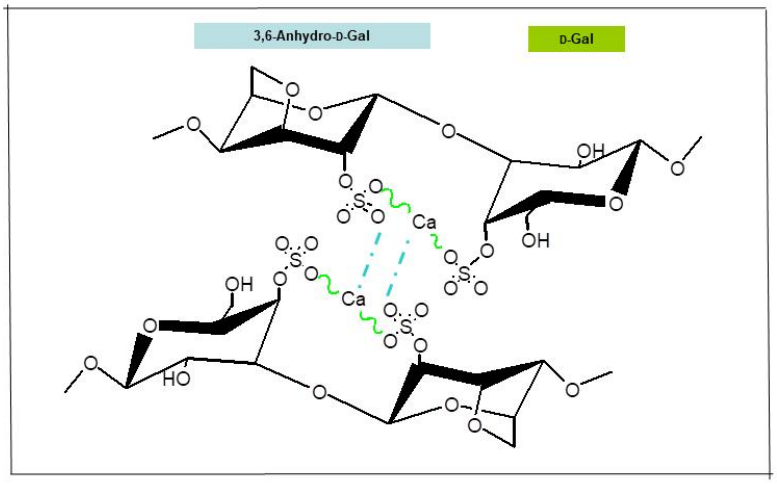

Figure 2. Intra- and intermolecular associations of ι-carrageenan molecules in aqueous solution. Associations: (∿∿) Ionic bonding; (– · –) electrostcit forces of attraction. (Reproduced with permission from references 14 and 15. Copyright 2014 and 2015 Scientific Research Publishing).

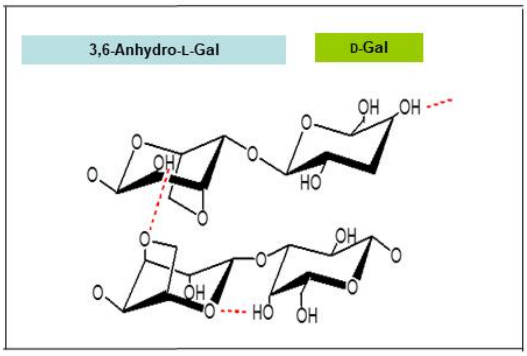

Figure 3. Intra- and intermolecular associations of agarose molecules.

The dotted red lines represent hydrogen bonding. (Reproduced with permission from references 14 and 15. Copyright 2014 and 2015 Scientific Research Publishing).

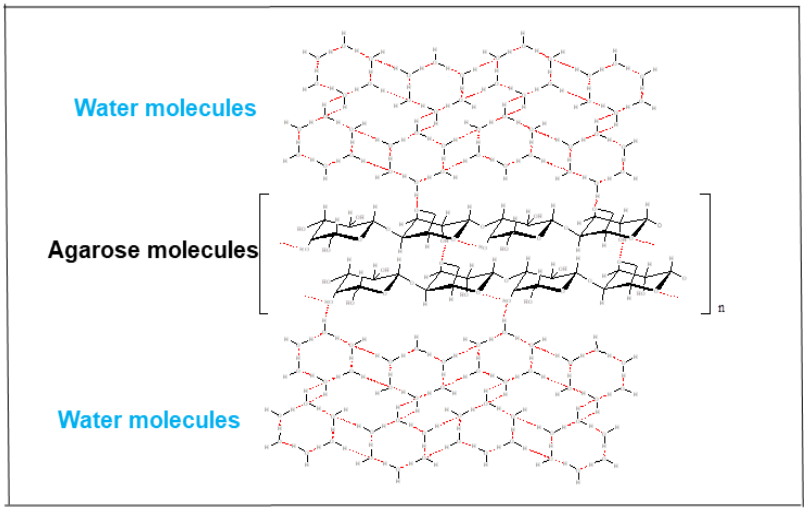

Figure 4. Gelling agarose in hexagonal water molecules.
The dotted red lines represent hydrogen bonding. (Reproduced with permission from references 14 and 15. Copyright 2014 and 2015 Scientific Research Publishing).

conformational transition of gelling agarose molecules occurs above the transition temperature (>60 °C) due to dissociation of intra- and inter-molecular hydrogen bonding.

We report herein the isolation and structural characterization of the κ-carrageenan-like agarose from *Gracilaria coronpifolia* which belong to red seaweed and grow in the coast of Okinawa Islands, Japan. The seaweed is used in traditional food as a gelling additive in Okinawa, Japan. This paper is the first to report sulfate groups substituted at C-4 of β-D-galactose residue on agarose molecule.

2. MATERIALS AND METHODS

2.1 Materials

Gracilaria coronpifolia was collected in Ishigaki Island, Okinawa Prefecture, Japan. The collected seaweed was washed with tap water and then air-dried at 40°C for 24 h. The dried seaweed was powdered using a crusher. The powder was stored in refrigerator (4°C) until extraction.

2.2 Polysaccharide Extraction

The powdered seaweed sample (3 g) was suspended in 500 mL of distilled water and

stirred at 100°C for 1 h. The suspension was filtered, and the filtrate was concentrated at 40°C using a rotary evaporator. Ethanol (2 vols.) was added to the concentrated solution to precipitate polysaccharide. The precipitate was washed with ethanol twice and then dried in a vacuum chamber at 40°C [3,4].

The dried precipitate was dissolved in distilled water and then filtered through Celite 545 (Nakarai, Japan)). The filtrate was passed through a column of Amberlite IR-120B (ø 5 × 30 cm, H^+ form). The eluate was adjusted to pH 7 with 0.05 M NaOH and concentrated using a rotary evaporator at 40°C. The concentrated solution was dialyzed against distilled water, and freeze-dried. The standard κ-carrageenan was prepared from *Hypnea charoides* [3] and prepared the same methods as described above [3,4].

2.3 Chemical Components Analysis

Total carbohydrate was determined by the phenol-sulfuric acid method using D-galactose as a standard [16]. Ash content was determined by incinerating the polysaccharide for 24 h in a muffle furnace at 550°C and then weighed the residue.

2.4 Methanolysis

The polysaccharide (10 mg) was treated with 0.5 M hydrogen chloride in anhydrous methanol at 105°C for 12 h in a sealed tube. The reaction mixture was neutralized with silver carbonate at 60 °C, and then filtered and evaporated [2-5].

2.5 Thin-layer Chromayography

Thin-layer chromatography was carried out on glass plate (20 cm in length) treated with silica gel and using a solvent of butanol-ethanol-water (4:1:5). Chromatograms were sprayed with 10% sulfuric acid in water and heated at 100°C for 15 min [2-5].

2.6 Fourier Transform Infrared (FTIR) Spectroscopy and Specific Rotation

FTIR spectrum was measured using a FTS-3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) in transmittance mode from 4000 to 400 cm^{-1} in KBr disc which was prepared by dispersing solid sample in the KBr salt [3,4].

The specific rotation of the polysaccharide was measured at 589 nm on a polaimeter (P-1010, Japan Spectroscopic Co., Ltd., Japan) for a 0.2% (W/V) solution in distilled water at room temperature [5,17].

2.7 ^{1}H- and ^{13}C-Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H- and ^{13}C-NMR spectra were recorded on a FT-NMR spectrometer (AVANCE III 400 MHz, Bruker, Co., Ltd, Germany) at 500.00 and 125.65 MHz. The purified polysaccharide (2.0%, W/V) was dissolved in D_2O and recorded at 70°C. The ^{1}H-and ^{13}C-NMR chemical shifts were expressed in parts per million (ppm) relative to internal sodium 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP, 0.00 ppm) as an internal standard [5,17].

2.8 Gel Preparation

Gel of the polysaccharide was prepared after dissolving the polysaccharide in distilled water at 0.2% (W/V) at room temperature and 13.5 mM KCl was added [1,3].

3. RESULTS

3.1 Polysaccharide Preparation from *Gracilaria coronopifolia*

One of red seaweed, *G. coronopifolia,* as shown in Fig. 5, which was collected in Ishigaki Island, Okinawa Prefecture, Japan, reached 10-20 cm long, having many branches, which was about φ 5.0-7.3 mm. The collected seaweeds were washed with tap water and then dried by an air-dried oven at 40 °C for 24 h. The polysaccharide was purified as described in Materials and Methods. The purified polysaccharide was a colorless, fibrous powder, with yield of 15.4% (w/w) based on dried seaweed.

3.2 Identification of Sugar Components of the Polysaccharide

The total carbohydrate, sulfuric acid and ash of the purified polysaccharide were 69.5% (W/W), 20.3 % and 24.3%.

The methanolysis product of the polysaccharide by thin-layer chromatography (Fig. 6) indicated the presence of 3,6-anhydro-methy-β-D-galactoside (spot a; weak), methyl-α-D-galactoside (b; strong), and methyl-β-D-galactoside (c; strong) [3-5].

Figure 5. Photograph of *Gracilaria coronopifolia* collected in Ishigaki Island, Okinwa, Japan.

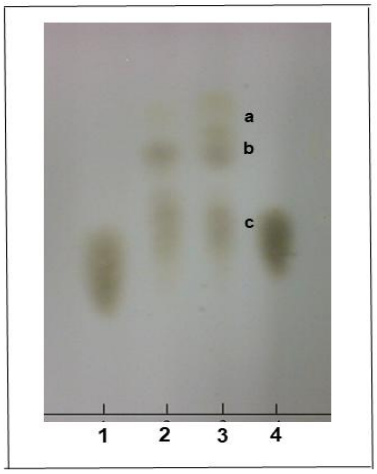

Figure 6. Thin-layer chromatogram of methanoyzate of the polysaccharide from *Gracilaria coronopifolia.*
1, α-D-Galactoside; 2, Methanolyzate of κ-carrageenan;
3, Methonolyzate of the polysaccharide from *Gracilaria coronopifolia*;
4, β-D-galactoside.

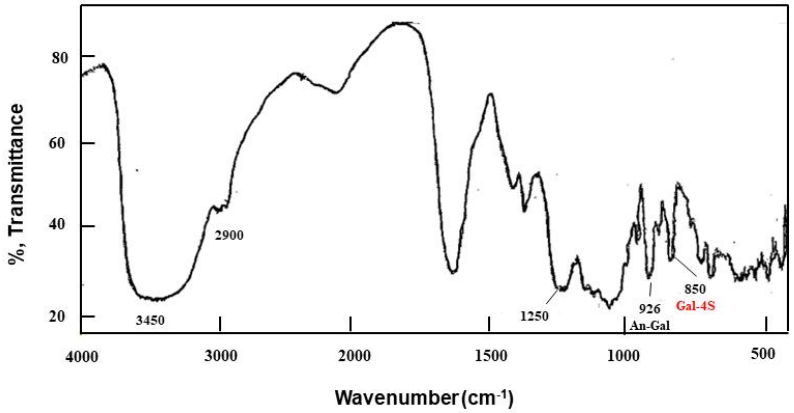

Figure 7. Infrared spectrum of the polysacchride isolated from *Gracularia coronopifolia*.

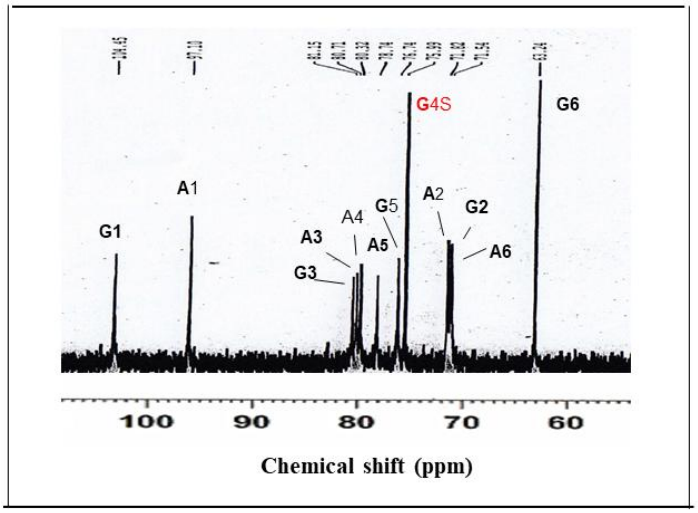

Figure 8. ^{13}C-NMR spectrum of the polysaccharide 70℃.

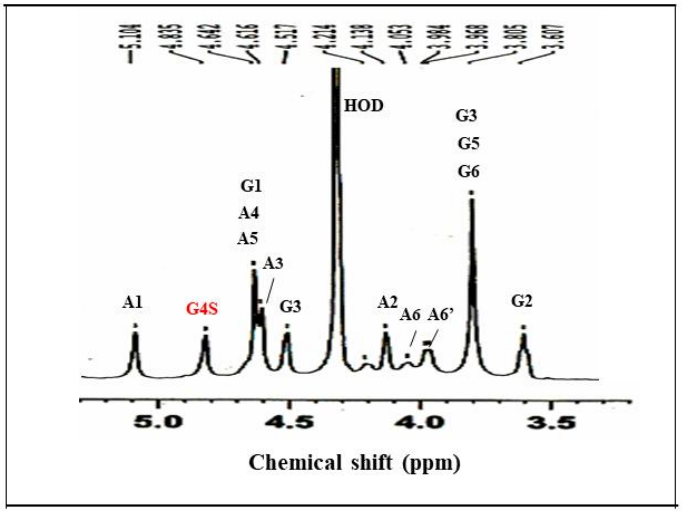

Figure 9. ^{1}H-NMR spectrum of the polysaccharide 70℃.

Table 1. Chemical shift of ^{1}H- and ^{13}C-NMR spectra of the polysaccharide isolated from *Gracilaria coronopifolia*.

Sugar residue	1C/1H	2C/2CH	3C/3CH	4C/4H	5C/5CH	6C /6H	/6H'
β-D-Galactose-4-sulfate	104.45/4.64 2	71.54/3.607	81.14/3.805	75.99/4.835	76.73/3.805	63.23/3.805	
α-3,6-Anhydro-D-or L-galactose	97.14/5.104	71.18/4.214	80.71/4.517	80.31/4.642	78.73/4.642	71.53/4.053	/3.984

3.3 FTIR Spectrum and Specific Rotation

Fig. 7 shows spectrum of the polysaccharide. A broad absorption at 1245 cm^{-1} was common to all the sulfated polysaccharides due to sulfate absorption [3-5]. An absorption at 850 cm (strong) was assigned to be ester sulfate on C-4 of 3-linked D-galactose residue [3-5,18,19]. An absorption at 939 cm^{-1} (weak) was assigned to C-O ether bond of 4-linked 3,6-anhydro-

Figure 10. Gelling polysaccharide at a concentration of 0.2% with addition of 13.5mM KCL isolated from *Gracilaria coronopifolia*.

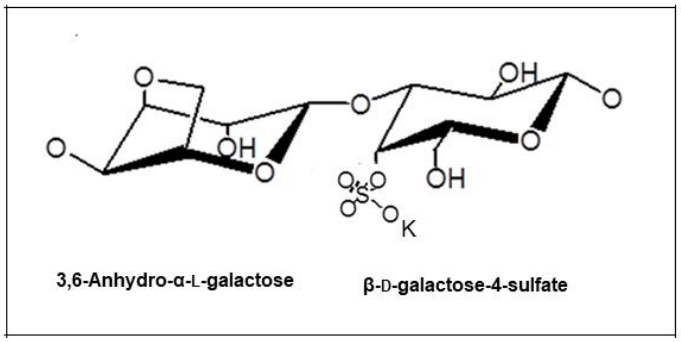

Figure 11. Chemical structure of the κ-carrageenan-like agarose isolated from *Gracilaria coronopifolia*.

galactose residue [3-5,18,19]. These data were consistent with those of published papers [3-5,18-20]. Although *Gracilaria* species are agar (agarose) source seaweed, the polysaccharide from *G. coronopifolia* substituted with sulfate group at C-4 of β-D-galactose residue.

The specific rotation $[\alpha]_{589}$ of the polysaccharide at 25 °C was estimated a value of -2.0 ° (c 0.2%, H_2O). The value was higher than agar (agarose) from *Gracilaria blodgetti* (-56°) [3], but lower than that of κ-carrageenan (+56.5°) [3].

3.4 ^{13}C- and ^{1}H-NMR Spectra Analysis

^{13}C-NMR spectrum of the polysaccharide is shown in Fig. 8 where 11 signals were observed. From published papers [18-23], the signal of β-D-galactopyranose at G1, 104.45; G2, 71.54; G3, 81.14; G4, 75.99; G5, 76.73; G6, 63.23 and 3,6-anhydro-α-D or L-galactopyranose at A1, 97.14; A2, 71.18; A3, 80.71; A4, 80.31; A5, 78.73; and A6, 71.53 ppm were assigned, where, G refers to D-galactose and A is 3,6-anhydro-D or L-galactose residue. These results consistent to those of published papers of κ-carrageenan [18-23] and agarose [12,20], except at C-4 of the latter molecules. The chemical shifts are summarized in Table 1.

^{1}H-NMR spectrum of the polysaccharide is well characterized and shown in Fig. 8. Ten signals were observed. From published papers [4,18,24,25], the signal of β-D-galactopyranose at G1, 4.64; G2, 3.61; G3, 3.31; G4, 4.83; G5, 3.81; G6, 3.81 and 3,6-anhydro-α-D or L-galactopyranose at A1, 5.10; A2, 4.14; A3, 4.64; A4, 4.64; A5, 4.64; A5, 4.64 and A6, 3.99 ppm

were assigned. These data were consistent to those of published κ-carrageenan [18,24,25,26] and agarose except at H-4 of the latter molecule [12,24,26,]. The results are summarized in Table 1.

3.5 Gel

The polysaccharide did not gelled at a concentration 0.5 % (W/V) in distilled water at room temperature (28 °C). However, as shown in Fig. 10, a weak and transparent gelation occurred in a solution of 0.2% with addition of 13.5 mM KCl. As reported proceeding papers, very strong and cloudy gel was observed in κ-carrageenan which isolated from *Hypnea chaloides* [3], while transparent and a little strong gel was observed in agarose (agar) from *Gracilaria blodgettii* [1]. Hoyle [27] reported that the gel strength of agar from *G. coronopifolia* was 1/3-1/4 of that of *G. bursapastoris*. Praiboon et al. reported that gel strength of sulfated agar isolated from *G. fisheri* (sulfate content, 4.54 %) and *G. edulis* (7.54%) were weak, but they increased after desulfation [28].

4. DISCUSSION

As *Gracilaria* species are major commercial source of agar (agarose), a number of studies have been carried out [1,2,29-32]. Indeed, we identified agar (agarose) and methylated agar (agarose) in the proceeding papers [1,2] and the thermostable agarose (agar) was discovered from *G. arcuata*. However, there is no structural report on the agar (agarose) from *G. coronopifolia* yet. In this investigation, a polysaccharide was isolated from the red seaweed which had high content of sulfate (20.3%) that was unexpected result. The polysaccharide consisted of β-D-galactose and 3,6-anhydro-α-D or L -galactose. Furthermore, IR spectrum of the polysaccharide indicated the presence of β-D-galactose-4-sulfate at 845 cm^{-1}, in addition to 3,6-anhydro-α-D or L-galactose at 925 cm^{-1}, which were in agreement with those of κ-carrageenan [2,18,19].

On the contrary, the specific rotation $[\alpha]_{589}$ of the polysaccharide was -2.0° that was disagreement with published κ-carrageenans (+56.5°) [3]. Whereas, the specific rotation of agar (agarose) from *G. blodgettii* [1] was reported as -56° and the methylated agar from *G. arcuata* was almost the same (-60°) [2], suggesting that the polysaccharide from *G. coronopifolia* seems to be an agar (agarose).

However, the ^{13}C- and ^{1}H-NMR spectra of the polysaccharide were in agreement with those of published κ-carrageenans [18-25] and essentially agreed with those of agar (agarose) except at C-4 and H-4 of β-D-galactose residue [12,20,24,26]. They were moved to higher chemical shift from 68.61 ppm [2] to 76.25 ppm for the ^{13}C-NMR spectra and from 4.13 [2] to 4.83 ppm for the ^{1}H-NMR spectra.

As reported previously [3], gelling κ-carrageenan from *Hypnea chaloides* at 0.2% with addition of 13.5 mM KCl was very strong and cloudy, but the polysaccharide from *G. coronopifolia* showed the transparent and very weak gel at the same conditions. Indeed, the gel was broken down 20 min after preparation at room temperature caused by dissociation of intra- and intermolecular electrostatic forces of attraction between K^+ and oxygen atoms (Fig. 1). As reported previously, a transparent and a little strong gel was observed in agar (agarose) from *G. blodgettii* at 0.2% without 13.5 mM KCl. Furthermore, the gel of agar (agarose) isolated from *G. coronopifolia* was reported to have 1/3 to 1.4 strong as compared with that of an agar from *G. burusapastoris* [27]. The weak gel may be due to the substitution of sulfate groups at C-4 of the β-D-galactose residue preventing the formation of the intramolecular hydrogen bonding on agar (agarose) molecules (see Fig. 3).

Results and discussion support the C-4 of β-D-galactose residues which adopt at axial configuration contribute to the intramolecular hydrogen bonding because gelation did not occur in.0.2% solution of the polysaccharide, but weak gel was obtained with addition of 13.5 mM KCl where intra- and intermolecular electrostatic forces of attraction having smaller binding energy than hydrogen bonding were built up through K^+-cations with different molecules. The weak gel (small amount of intra- and intermolecular electrostatic forces of attraction) might be caused by enantiomer of 3,6-anhydro-α-D-galactose residues in the presence of 13.5 mM KCl. The chemical structure of the polysaccharide was illustrated in Fig. 11.

5. CONCLUSION

Thus, we conclude that the polysaccharide isolated from *Gracilaria coronopifolia* is the κ-carrageenan-like agarose. Although, 6-*O*-sulfated agarose was reported from *Gloiopeltis furcata* [33], this study is the first to report on κ-carrageenan-like (4-*O*-sulfated) agarose.

ACKNOWLEDGEMENTS

The authors thank Mr. Wen Jiang for his technical assistance.

Tako, wishes to thank the late Professor Danji Nomura his supervisor in the Doctoral Course in Food Science and Technology of Kyushu University for encouraging discussions about Nobel Laureate Hans von Euler-Chelpin (Chemistry, 1929). Emil Fischer, van't Hoff, Max Planck, Hermann Nernst and Svante Arrhenius were influential in this work as professors of von Euler at Berlin, Gottingen and Stockholm Universities.

Professor Nomura was also encouraging Tako talking about Dr. Masuzo Shikata Professor of Kyoto University and Umetaro Suzuki Professor of the University of Tokyo and Institute of Physical and Chemical Research.

The study of the structure-function relationship of polysaccharides first began when listening to the First Movement of Piano Sonata No. 14 Op. 27-2 "Moon Light" composed by Ludwig van Beethoven on July 16th, 1973 when Tako was a graduate student of Doctoral Course in Food Science and Technology of Kyushu University, Fukuoka, Japan.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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