トゲキリンサイから□□カラギーナンの分離・同定

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Isolation and Characterization of ι-Carrageenan from *Eucheuma serra* (Togekirinsai)*

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A gelling polysaccharide was extracted from Togekirinsai (*Eucheuma serra*), which was collected from Miyako Island, Okinawa Prefecture, Japan, and purified by gelation with calcium chloride. The purified polysaccharide obtained from Togekirinsai was colorles fibrous powder; yield, 38.3% (w/w) based on dried seaweed and 4.6% (wet seaweed). The total carbohydrate, ash and moisture contents of the polysaccharide were 71.4, 21.2 and 7.1%, respectively. The content of total sulfate was estimated to be 23.8%. The polysaccharide was composed of D-galactose, 3,6-anhydro-D-galactose and ester sulfate at a molar ratio of 1.2: 1.0: 1.5. Molecular mass of the polysaccharide was estimated to be about 2.8×10^5. The infrared spectrum and values of optical rotation of the polysaccharide at different temperatures were in agreement with those of standard ι-carrageenan. The ^{13}C- and ^1H-NMR spectra showed the polysaccharide isolated from Togekirinsai was composed of D-galactopyranosyl-4-sulfate and 3,6-anhydro-D-galactopyranosyl-2-sulfate. These results indicate that the gelling polysaccharide is a ι-carrageenan.

ι-Carrageenan is found in certain species of red seaweed (*Rhodophyceae*) and is prepared by selective precipitation with calcium chloride. The composition and properties of ι-carrageenan is very close to κ-carrageenan. It has the same carbohydrate chain built with κ-carrageenan, that is the position of sulfate located at C-4 of 3-*O*-linked β-D-galactopyranose residues, but differs from ι-carrageenan in the position of sulfate groups located at C-2 of 4-O-linked 3,6-anhydro-α-D-galactopyranose residues.[1,2] Polysaccharides, such as agar, fucoidan and κ-carrageenan, were isolated from Kubireogonori (*Gracilaria blodgettii*),[3] Yumigata-ogonori (*Gracilaria arcuata*),[4] Okinawamozuku (*Cladosiphon okamuranus*),[5] Itomozuku (*Nemacystus decipiens*)[6] and Ibaranori (*Hypnea charoides*),[7] which were harvested from the Okinawa Islands. However, ι-carrageenan has never been isolated from any Okinawa seaweeds.

Togekirinsai (*Eucheuma serra*), which also belongs to red seaweed (*Rhodophyceae*), is grown on Miyako Island of Okinawa Prefecture and has been used as a gelling additive of "Urusu" since long ago. Recently, because this seaweed is also used as salad dressing, its utilization in the food industry is on the increase. The polysaccharide from Togekirinsai (*Eucheuma serra*) has not been well studied yet.

We report herewith the isolation and characterization of the gelling polysaccharide from Togekirinsai.

MATERIALS AND METHODS

Materials. Togekirinsai (*Eucheuma serra*) used in this study was collected in July 1999 from Miyako Island, Okinawa. The collected seaweed was washed with tap water and then dried by an air-dried oven at 40°C for 24 h. ι-Carrageenan, used as the standard, was extracted from *Eucheuma spinosum* supplied by Taiyo Kagaku Co., Ltd., Japan.

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Extraction and purification of polysaccharide. An air-dried seaweed sample (5 g) was suspended in water and heated on a heater with distilled water (500 mL) at 100°C for 2 h to extract the polysaccharide. The extract was then centrifuged at 5000 rpm for 20 min and the supernatant was filtered through a suction filter (Celite 545). The precipitate was then dialyzed against distilled water overnight to complete the dissolution. The solution was diluted with distilled water to 500 mL, and filtered through a suction filter (Celite 545) again. The extract was concentrated in an evaporator to about half of the original volume. In the presence of calcium chloride (0.2%, 20 mL), ethanol (2 vols) was added to the solution. The precipitate was dried *in vacuo*. The crude polysaccharide (2.3 g) was dissolved in 300 mL of distilled water and filtered through a suction filter (Celite 545). The filtrate was dialyzed against distilled water until free from chloride, and then ethanol (2 vols) was added to the dialyzate. The precipitate was dried *in vacuo*.

Acid hydrolysis. The purified polysaccharide (50 mg) was dissolved in distilled water (20 mL) at 80°C and sulfuric acid was added to a final concentration of 0.4 M. The mixture was then heated at 100°C for 3 h. The hydrolyzate was neutralized with $BaCO_3$.

Determination of total carbohydrate, D-galactose and 3,6-anhydro-D-galactose. The total carbohydrate was determined by the phenol-sulfuric acid method.[8] D-Galactose and 3,6-anhydro-D-galactose were determined by the methods of cysteine-hydrochloric acid[9] and resorcinol,[10,11] respectively.

Estimation of ester sulfate. Purified polysaccharide (40 mg) was dissolved in distilled water (20 mL) at 80°C and hydrochloric acid was added to the solution to a final concentration of 1.0 M HCl. The mixture was then heated at 110°C for 3 h. Two milliliters of the hydrolyzate was applied to a liquid chromatograph, DM 500 (Dionex Co., Ltd., USA), on a column (AS 4 A-SC 4×250 mm) equilibrated with 1.7 mM $NaHCO_3$×1.8 mM Na_2CO_3. The chromatography was carried out at a flow rate of 1.5 mL/min at 35°C.

Methanolysis. The polysaccharide (10 mg) was treated with 0.5 M hydrogen chloride in 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.[12]

Paper chromatography, liquid chromatography and thin-layer chromatography. Paper chromatography was performed on Advantec Filter Paper No. 50 using a solvent of butanol–ethanol–water (4: 1: 5). Chromatograms were sprayed with aniline hydrogen phthalate in butanol saturated with water and heated at 105°C for 15 min.

Two milliliters of the hydrolyzate was applied to a liquid chromatograph, DM 500 (Column; Carbo. PacPAL 4×250 mm, Dionex Co., Ltd.), equilibrated with 5 mM NaOH. The chromatography was carried out at a flow rate of 1 mL/min at 35°C.

Thin-layer chromatography was carried out on glass plate (20 cm in length) treated with silica gel containing calcium sulfate as the binder and using a solvent of butanol–ethanol–water (4: 1: 5). Chromatograms were sprayed with 10% sulfuric acid in water and heated at 110°C for 15 min.

Molecular mass determination. The molecular mass of the polysaccharide was determined by high-performance liquid chromatography (HPLC) (Shimadzu SCL-6 B; Shimadzu Seisakusho, Co. Ltd., Japan) on a Superdex 200 column (TSK gel G 4000 PW×L) with a sample loop of 200 μL. The HPLC operation was performed at room temperature. The column was developed with 50 mM phosphoric acid buffer, and the same buffer supplemented with 150 mM sodium chloride and fractions were collected at a flow rate of 0.2 mL/min. The fractions were treated by the phenol-sulfuric acid reagent, and color development was measured at 490 nm. Standard pullulan (P-82), P-400 (molecular mass, 4.0×10^5), P-100 (1.0×10^5), P-20 (2.0×10^4), and P-5 (0.5×10^4), Pharmacia Chemicals Co., Ltd., Sweden, having definite molecular mass were used for calibration.

The molecular mass of the polysaccharide was also determined from the intrinsic viscosity at 25°C in 0.1 M NaCl according to the relationship $[\eta] = 3.1 \times 10^{-3} \cdot M^{0.95}$, determined for ι-carrageenan.[13] The intrinsic viscosity $[\eta]$ was determined by measuring the specific viscosity with an

Ostwald-type viscometer.

Measurements of infrared spectra and optical rotation. Infrared spectra were recorded with an infrared spectrophotometer (IR-8200, Japan Spectroscopic Co., Ltd., Japan) for samples dispersed in KBr discs.

Optical rotation was measured at 589 nm on a polarimeter (DIP-180, Japan Spectroscopic Co., Ltd., Japan) for a 0.1% (w/v) solution in distilled water.

^{13}C- and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. The ^{13}C- and ^{1}H-NMR were recorded at 80°C on a FT-NMR spectrometer (JNM α500, Nihon Denshi, Co., Ltd., Japan). The chemical shifts were expressed in parts per million (ppm) relative to internal dimethyl sulphoxide (DMSO), 39.6 ppm for ^{13}C-NMR and 2.697 ppm for ^{1}H-NMR, respectively. The polysaccharide isolated from Togekirinsai (80 mg) and standard ι-carrageenan were dissolved in 4 mL of D_2O at 80°C for 30 min and recorded at 80°C.[14-17]

RESULTS

Preparation of polysaccharide from Togekirinsai.

Figure 1 shows a photograph of Togekirinsai (*Eucheuma serra*) which was collected in July from Miyako Island of Okinawa Prefecture, Japan. The Togekirinsai reached 15–20 cm long having the shape of a thorny branch, the thick part of which was about 5.0 mm and its tip was sharp.

The collected seaweed (2000 g) was washed with tap water and then dried by an air-dried oven at 40°C for 24 h. The weight of the air-dried seaweed decreased to 240 g. The gelling polysaccharide was prepared and purified as described in MATERIALS AND METHODS. The purified polysaccharide obtained from Togekirinsai was a colorless, fibrous powder, with a yield of 38.3% (w/w) based on dried seaweed and 4.6% (wet seaweed).

Chemical components of the polysaccharide.

The total carbohydrate and ash of the purified polysaccharide were estimated to be 71.4 and 21.2%, respectively. As shown in Table 1, the moisture of the purified polysaccharide was estimated to be 7.1%. The sulfuric acid was estimated to be 23.8% by HPLC after hydrolysis of the polysaccharide. The 3,6-anhydro-D-galactose and D-galactose were estimated to be 30.5 and 39.1%, respectively, by colorimetric determination with cys-

Table 1. Chemical components of purified polysaccharide from *Eucheuma serra*.

% (w/w)

	D-Galactose	3,6-Anhydro-galactose	H_2SO_4	Moisture
Polysaccharide	39.1	30.5	23.8	7.1

Fig. 1. Photograph of *Eucheuma serra* (Togekirinsai).

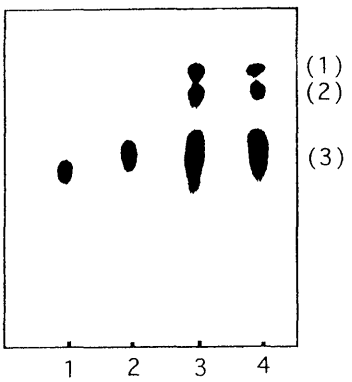

Fig. 2. Thin-layer charomatogram of methanolyzate of the polysaccharide isolated from *Eucheuma serra*.

1. methyl-β-D-galactoside; 2, methyl-α-D-galactoside; 3, methanolyzate of the polysaccharide from *Eucheuma serra*; 4, methanolyzate of standard ι-carrageenan. (1), 3,6-anhydro-methyl-β-D-galactoside; (2), 3,6-anhydro-methyl-α-D-galactoside; (3), methyl-β- and -α-D-galactose. Experimental conditions are described in the text.

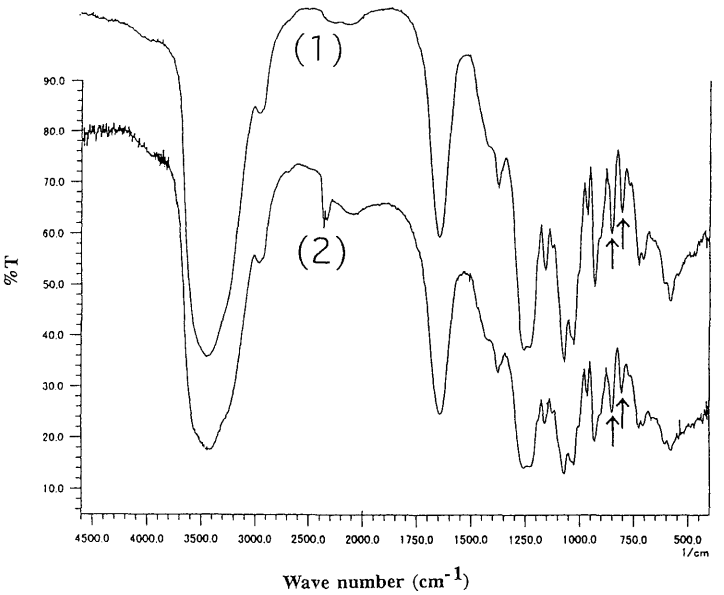

Fig. 3. Infrared spectra of the polysaccharide from *Eucheuma serra* and ι-carrageenan standard.

(1), polysaccharide from *Eucheuma serra*; (2), ι-carrageenan standard. Experimental conditions are described in the text.

Table 2. Optical rotation of the polysaccharide extracted from *Eucheuma serra*.

Temperature (°C)	60	50	40	30	25	20	10
Togekirinsai	+0.092	+0.096	+0.102	+0.102	+0.106	+0.118	+0.142
ι-Carrageenan	+0.096	+0.098	+0.103	+0.104	+0.107	+0.119	+0.144

teine-hydrochloric acid-resorcinol reagents.

Identification of sugar components of the polysaccharide.

A paper chromatogram (data not shown) of the hydrolyzate of the purified polysaccharide and ι-carrageenan used as the standard showed spots identical to D-galactose. Only one peak was also shown on a liquid chromatogram, and it was identical to D-galactose. As shown in Fig. 2, examination of the methanolysis product of the polysaccharide by thin-layer chromatography indicated the presence of 3,6-anhydro methyl β-D-galactoside at the position of spot 1, 3,6-anhydro methyl-α-D-galactoside at the position of spot 2,[18] and a mixture of methyl-α- and β-D-galactoside at the position of spot 3.

Determination of molecular mass.

The molecular mass of purified polysaccharide isolated from Togekirinsai was measured by the gel chromatography method on a superdex 200 colunm. According to the standard calibration curve obtained from the definite molecular mass pullulan, the molecular weight of this purified polysaccharide was calculated to be approximately 2.8×10^5. The molecular mass was also calculated to be about 2.8×10^5 by the viscometric method with an Ostwald-type viscometer.

Infrared spectra and optical rotation of the polysaccharide.

The infrared spectrum of the polysaccharide (Fig. 3) showed a broad band at 1240–1250 cm^{-1}, and was common to all of the sulfated polysaccharides due to sulfate absorption.[19,20] The peak at 805 cm^{-1} was assigned to be a ester sulfate on C 2 of

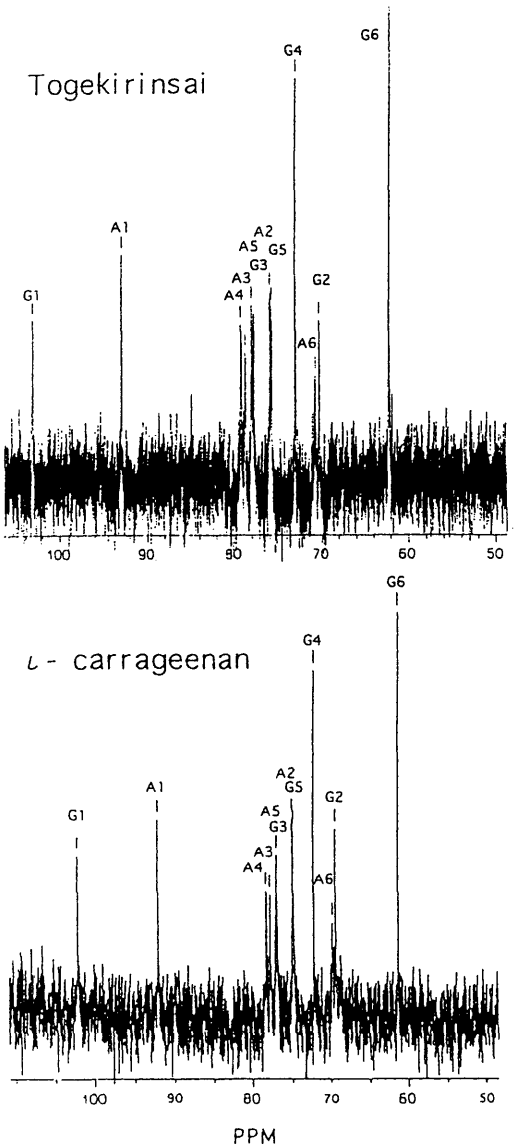

Fig. 4. ^{13}C-NMR spectrum of the polysaccharide from *Eucheuma serra* and standard *Eucheuma spinosum* at 80°C.
Details are described in the text.

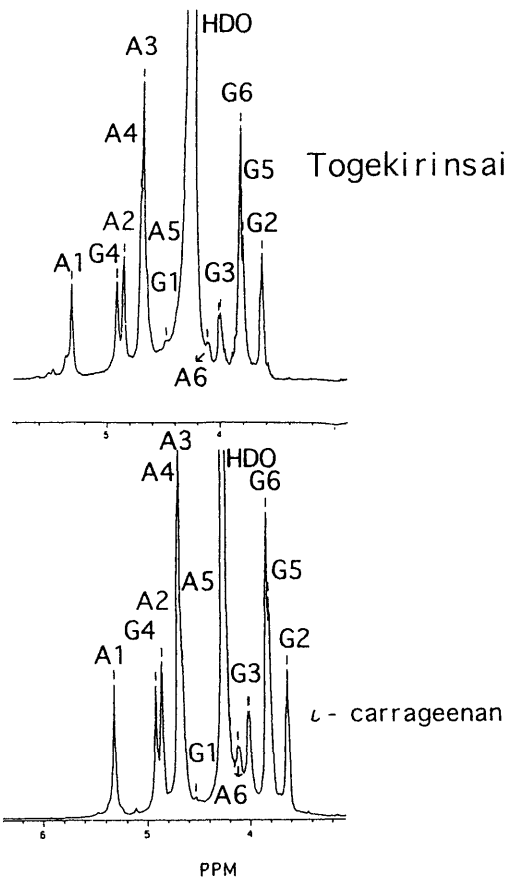

Fig. 5. ^{1}H-NMR spectrum of the polysaccharide from *Eucheuma serra* and standard *Eucheuma spinosum* at 80°C.
Details are described in the text.

Table 3. Twelve chemical shifts of the ^{13}C-NMR spectrum of the purified polysaccharide isolated from Togekirinsai and the standard *Eucheuma spinosum*.

		^{13}C Chemical					
Solvent: D_2O		C-1	C-2	C-3	C-4	C-5	C-6
Togekirinsai	Galactose (G)	104.6	69.7	77.2	72.5	75.2	61.7
	Anhydro-galactose (AG)	94.4	77.4	80.2	80.7	79.4	72.3
ι-Carrageenan (standard)	Galactose (G)	102.3	69.4	77.0	72.2	75.0	61.3
	Anhydro-galactose (AG)	92.2	74.8	77.8	78.3	77.1	69.8

Table 4. Twelve chemical shifts of the ^{1}H-NMR spectrum of the purified polysaccharide isolated from Togekirinsai and the standard *Eucheuma spinosum* (supplied by Taiyo Kagaku Co., Ltd.) at a concentration of 2.0% (w/v).

		^{1}H Chemical shifts					
Solvent: D_2O		H-1	H-2	H-3	H-4	H-5	H-6
Togekirinsai	Galactose (G)	4.481	3.640	3.999	4.910	3.805	3.828
	Anhydro-galactose (AG)	5.314	4.849	4.677	4.701	4.648	4.116
ι-Carrageenan (standard)	Galactose (G)	4.482	3.638	4.007	4.918	3.810	3.837
	Anhydro-galactose (AG)	5.320	4.856	4.687	4.687	4.652	4.108

the 4-linked 3,6-anhydro-D-galactose and the peak at 845 cm^{-1} to be ester sulfate on C 4 of the 3-linked-D-galactose unit, and 930 cm^{-1} showed 3,6-anhydro-D-galactose.[19–25] These data were consistent with those of standard ι-carrageenan prepared from *Eucheuma spinosum*.

The optical rotation of the purified polysaccharide at various temperatures is summarized in Table 2. It showed a value of +0.092° at 60°C and then increased gradually with decreasing temperature. The values were also in agreement with those of standard ι-carrageenan at various temperatures.

^{13}C- and ^{1}H-nuclear magnetic resonance (NMR) spectra analysis.

As shown in Fig. 4, at 80°C, in D_2O, the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of the Ca salt of the polysaccharide isolated from Togekirinsai at a concentration of 2.0% (w/v) and commercial Ca salt of ι-carrageenan prepared from *Eucheuma spinosum* supplied by Taiyo Kagaku Co., Ltd. show 12 major peaks. The chemical shifts were estimated at G 1, 104.6; G 2, 69.7; G 3, 77.2; G 4, 72.5; G 5, 75.2; G 6, 61.7; A 1, 94.4; A 2, 77.4; A 3, 80.2; A 4, 80.7; A 5, 79.4; and A 6, 72.3 ppm, respectively; here, G refers to D-galactose and AG is the 3,6-anhydro-D-galactose residue. These data were consistent to those of standard ι-carrageenan which showed the chemical shifts at 104.6 ppm (C 1 of 3-linked β-D-galactopyransoyl-4-sulfate), 94.4 ppm (C 1 of 4-linked 3,6-anhydro-α-D-galactopyransoyl-2-sulfate), 72.5 ppm (C 4 of 3-linked β-D-galactopyransoyl-4-sulfate), 69.7 (C-2), 77.2 (C-3), 75.2 (C-5) and 61.7 ppm (C-6) of D-galactose, and 77.4 (C-2) 80.2 (C-3) 80.7 (C-4) 79.4 (C-5) and 72.3 ppm (C-6) of 3,6-anhydro-galactose, respectively (Table 3). These chemical shifts are assigned to the repeating unit of ι-carrageenan.[2,14–17,23,26,27] No other chemical shift assigned to the carbon atom of the sulfate groups substituted at any carbon atoms in the D-galactose or 3,6-anhydro-D-galactose was estimated. This means that the polysaccharide isolated from Togekirinsai was composed of D-galactose-4-sulfate and 3,6-anhydro-D-galactose-2-sulfate.

The proton chemical shift of the polysaccharide isolated from Togekirinsai is shown in Fig. 5. The ^{1}H-NMR spectrum of the Ca salt of the polysaccharide and commerical Ca salt of the ι-carrageenan prepared from *Eucheuma spinosum* supplied by Taiyo Kagaku Co., Ltd. show nine major peaks, with the chemical shifts at 4.649, 3.640, 3.999, 4.910, 3.805, and 3.828 ppm corresponding to G 1, G 2, G 3, G 4, G 5, and G 6, respectively. The chemical shifts values of 5.314, 4.849, 4.677, 4.7012, 4.648, and 4.116 ppm correspond to A 1, A 2, A 3, A 4, A 5, and A 6, respectively. The coalescing signals of A 3, almost together with A 5, appear as one peak with a maximum at 3.805 ppm (Table 4). These data were in agreement with those of standard ι-carrageenan. As shown in Fig. 5 and Table 4, these chemical shifts of the polysaccharide isolated from Togekirinsai are assigned to the repeating unit of ι-carrageenan.[17,24] This means that the polysaccharide isolated from Togekirinsai is composed predominantly of ι-carrageenan.

DISCUSSION

Quantitative determination showed that the purified polysaccharide from *Eucheuma serra* (Togekirinsai) contains 39.1% D-galactose, 30.5% 3,6-anhydro-D-galactose and 23.8% sulfate group. Paper and liquid chromatographic analyses of the complete acid hydrolysis showed only D-galactose, and 3,6-anhydro-D-galactose was not found; the latter probably being destroyed during acid hydrolysis. The 3,6-anhydro-α- and -β-methyl galactosides were detected by thin-layer chromatography after methanolysis of the polysaccharide.

It is well-known that sulfate groups in the carrageenan molecules have been deduced by infrared spectra based on empirical assignment.[25] Indeed, the spectra obtained in this experiment may be useful in comparative studies of carrageenans. There were no differences in the basic structure units between the present polysaccharide and standard ι-carrageenan specimen so far as the infrared spectra were concerned. The values of optical rotation of the purified polysaccharide were also almost same as the standardized ι-carrageenan specimen at various temperatures. Furthermore, the data of ^{13}C- and ^{1}H-NMR spectra of the Ca salt of purified polysaccharide isolated from Togekirinsai at a concentration of 2.0% (w/v) were almost the same as those of the standard ι-carrageenan. The polysaccharide isolated from Togekirinsai (*Eucheuma serra*) was composed of D-galactopyranosyl-4-salfate, 3,6-anhydro-D-galactopyranosyl-2-sulfate and ester sulfate at a molar ratio of 1.2: 1.0: 1.5.

Thus, the results indicated that the sulfated polysaccharide from *Eucheuma serra* (Togekirinsai), which was purified by selective precipitation with calcium chloride, was a ι-carrageenan.

Commercial κ- and ι-carrageenans from some red seaweeds are obtained at 30–60% based on the dry algae.[13,22] The polysaccharide characterized as a ι-carrageenan was about 38.3% based on the dried algae. Thus, Togekirinsai (*Eucheuma serra*), an industrially important seaweed in Okinawa Prefecture, contains a high concentration of a ι-carrageenan.

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トゲキリンサイから ι-カラギーナンの分離・同定

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トゲキリンサイから ι-カラギーナンを分離・同定した．トゲキリンサイは沖縄県宮古島の内海で採取し，水洗いと 塩抜きの後，通風乾燥 (40℃, 24 時間) させ，乾燥藻体を得た．乾燥藻体を蒸留水に分散させ，煮沸によって多糖を抽出し，常法により精製すると，4.6% (対湿潤藻体) の収率で多糖を得た．本多糖の全糖量，灰分，硫酸はそれぞれ，71.4%，21.2% および 23.8% であった．本多糖を加水分解およびメタノリシスの後，ペーパークロマトグラフィー，液体クロマトグラフィーおよび薄層クロマトグラフィーにより，D-ガラクトースと 3,6-アンヒドロ-D-ガラクトースを同定した．また，システイン-硫酸法とレゾルシン法により，それぞれの糖の含量は 39.1% および 30.5% であった．本多糖の分子量はおよそ 28 万と推定された．また，本多糖の赤外吸収スペクトルおよび旋光度の結果は標品の ι-カラギーナンのそれらと良く一致した．さらに，^{13}C-および ^{1}H-NMR スペクトルの結果から，D-ガラクトース-4-硫酸と 3,6-アンヒドロ-D-ガラクトース-2-硫酸を同定した．以上の結果から，本多糖は ι-カラギーナンであると同定した．