Molecular Origin of the Rheological Characteristics of ι-Carrageenan Isolated from Togekirinsai (*Eucheuma serra*)

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The rheological properties of ι-carrageenan isolated from Togekirinsai (*Eucheuma serra*) were measured with a rheogoniometer. The flow curves of a Ca-salt of ι-carrageenan solution showed plastic behavior and the yield value was estimated to be 0.4, 1.7 and 7.7 Pa at 0.1, 0.2 and 0.3% (w/v) concentration, respectively. The dynamic modulus of Ca-salt of ι-carrageenan increased with increase in concentration and gelation occurred at a concentration of 0.3% (w/v) at room temperature. The Ca-salt showed larger values than did of Na- and K-salts of ι-carrageenan in dynamic viscoelasticity. The Na- and K-salts of ι-carrageenan had very large values in the presence of $CaCl_2$ (6.8 mM) in dynamic modulus which maintained a constant value as the temperature increased to 40°C. A transition temperature, at which dynamic modulus decreased rapidly, was observed at 40°C. The Ca-salt of ι-carrageenan decreased with the addition of urea (4.0 M). The gel formation of the ι-carrageenan isolated from Togekirinsai might be essentially attributed to intra- and intermolecular associations, contributed by sulfate groups of adjacent D-galactose and 3,6-anhydro-D-galactose residues through Ca^{2+} bridges with ionic bonding and attractive electrostatic forces within and between molecules.

Keywords : Togekirinsai, *Eucheuma serra*, ι-carrageenan, Ca-bridges, ionic bonding, molecular origin

ι-Carrageenan is found in certain species of red seaweed (Rhodophyceae) and the chemical composition is very close to κ-carrageenan. It is well known that ι-carrageenan consists of an alternating disaccharide repeating unit of (1→3)-linked-β-D-galactopyranosy1-4-sulfate and (1→4)-linked-3,6-anhydro-α-D-galactopyranosy1-2-sulfate residues (Rees, 1969; Dawes, 1977). ι-Carrageenan can be extensively used in the food, pharmaceutical, and cosmetic industries as a gelling agent (such as jelly), thickening (such as a low calorie beverage), texture enhancement (such as custard) and stabilizer (such as mousse) (Yamada, 2000). Many physical techniques have been used to investigate the effect of cations on the conformation and conformational ordering of carrageenan molecules (Arnott *et al.*, 1974; Morris & Chilvers, 1981; Michel *et al.*, 1997). Arnott *et al.* (1974) have reported the X-ray diffraction patterns obtained from well-oriented and highly crystalline Ca^{2+}, Sr^{2+} and Mg^{2+} salts of ι-carrageenan. These results establish the structure of the ι-carrageenan double helices as well as providing a detailed picture of the interaction between the divalent cations (Morris & Chilvers, 1981; Michel *et al.*, 1997) and the polysaccharide chains. Tako *et al.* (1987) have discussed the molecular origin for its rheological characteristics and proposed the possible mode of intra- and inter-molecular cation-selective bridges of ι-carrageenan in aqueous solution, as illustrated in Fig. 1. The intra-molecular Ca^{2+}

bridges may take place between sulfate groups substituting on anhydro-D-galactose and D-galactose residues. Each cation is coordinated with two sulfate groups with ionic bonding.

The bridge proposed has been developed into an intermolecular association mechanism of the Ca salt of ι-carrageenan in aqueous solution. As Brownian motion and kinetic energy (Abeysekera, 1993) of solvent and polymer molecules decrease upon cooling, intermolecular Ca^{2+} bridges may also occur on different molecules having attractive electrostatic forces. The oxygen bond of the sulfate groups at C-2 of the anhydro-D-galactose residues may contribute to the intermolecular Ca^{2+} bridges by cage effect (Tako *et al.*, 1987).

Many kind of seaweeds harvested from nature and mariculture have been reported in abundance on the shore of the Okinawa Sea, Japan. Some of these seaweeds have been cited as possible sources for industrial use while others are valuable foods for daily life. Agar (Tako, 1994; Tako *et al.*, 1999a), Fucoidan (Tako *et al.*, 1999b, 2000) and κ-carrageenan (Qi *et al.*, 1997a, 1997b) have been isolated and characterized from *Gracilaria blodgettii, Gracilaria arcuata, Nemacystus decipiens* (Itomozuku), *Cladosiphon okamuranus* and *Hypnea charoides*, respectively. Here, the genus *Eucheuma* has become on important commercial source of carrageenan owing to its abundance and successful cultivation (Lynch & Mulvihill, 1996; Yamada, 2000). However, ι-carrageenan has been investigated and used less than that of κ-carrageenan. Togekirinsai (*Eucheuma serra*) which belongs to the family of red seaweed (Rhodophyceae), is grown on Miyako Island of Okinawa Prefecture and has long been used as a gelling agent of a traditional food for daily life called "Urusu" (article of
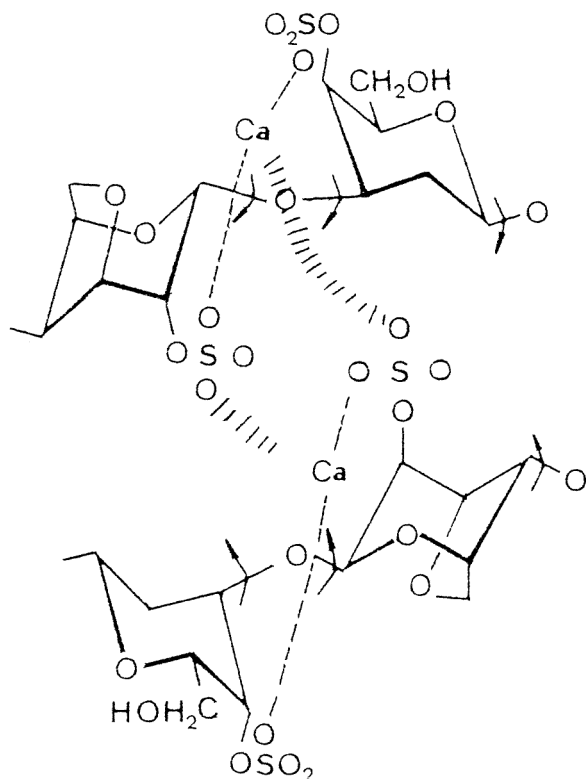
Fig. 1. Possible mode of the intra- and inter-molecular Ca^{2+} bridges of Ca salt of ι-carrageenan. Dotted lines refer to the ionic bonding and broken lines to the electrostatic force of attraction. Arrows indicate the orientation of the conformational angles.

diet). Recently, because this seaweed is also used in salad dressing, its utilization in the food industry is on the increase. The polysaccharide of Togekirinsai (*Eucheuma serra*) has not yet been well studied; therefore, we previously reported the chemical characterization of its gelling polysaccharide (Lin *et al.*, 2000). The infrared spectrum and values of optical rotation of the polysaccharide from Togekirinsai at different temperatures were in agreement with those of standard ι-carrageenan. The ^{13}C- and ^{1}H-NMR spectra showed the isolated polysaccharide was composed of D-galactopyranosyl-4 sulfate and 3,6-anhydro-D-galactopyranosyl-2-sulfate. The molar ratio of D-galactose, 3,6-anhydro-D-galactose and ester sulfate of ι-carrageenan was 1.2 : 1.0 : 1.5. The molecular mass of the polysaccharide was estimated to be about 2.8×10^5 (Lin *et al.*, 2000).

In this work, the non-Newtonian behavior and dynamic modulus of the ι-carrageenan aqueous solution from Togekirinsai and its rheological properties were analyzed with respect to its association characteristics in comparison with those of commercial ι-carrageenan.

Materials and Methods

Materials Togekirinsai (*Eucheuma serra*) used in this study was collected in July 1999 at Miyako Island, Okinawa. The collected seaweed was washed with tap water and then dried in an air-dried oven at 40°C for 24 h.

Preparation of calcium (Ca), potassium (K) and sodium (Na)-salt of ι-carrageenan Togekirinsai, an air-dried sample of the seaweed (5 g) was 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 at 8°C for 20 min and the supernatant was filtered through a suction filter (Celite 545, Wako, Co., Ltd., Osaka). The precipitate was then dialyzed against distilled water over night at 8°C to complete the dissolution. The solution was diluted with distilled water to 500 ml, and filtered again through a suction filter (Celite 545). The extract was heated and concentrated in a water bath (60°C) with an evaporator to about half of the original volume. In the presence of $CaCl_2$ (0.2%, 20 ml), ethanol (2 vols, 500 ml) was added to the solution. The precipitate was dried *in vacuo* (Anderson *et al.*, 1973). 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 at 8°C, and ethanol (2 vols, 500 ml) was then added to the precipitate. The precipitate was dried *in vacuo* (2.0 g).

Purified ι-carrageenan was redissolved in distilled water as a 0.5%, the solution was de-ionized by passage through a column of Amberlite 120 (H^+) cation-exchange resin, and neutralized at pH 7.0 with 50 mM $Ca(OH)_2$, KOH or NaOH to make the Ca-, K- and Na-salts, respectively. The solution was concentrated in an evaporator to about half of the original volume and then filtered through a suction filter (Celite 545). Ethanol (2 vols) was added to the filtrate and the precipitate was dried *in vacuo*.

Atomic absorption spectrophotometry ι-Carrageenan isolated from Togekirinsai (50 mg) was heated on a heater with nitric acid (25 ml, HNO_3) for 30 min by wet combustion method; it was heated again with 60% (w/v) perchloric acid (15 ml) for 10 min, filtered through a filter paper and distilled water added to a volume of 100 ml. Atomic absorption was measured with a flame spectrophotometer (AA-660, Shimazu Seisakusho Co., Ltd., Tokyo).

Methods

Viscosity and dynamic viscoelasticity measurements Viscosity at various shear rates (1.19–76.02 s^{-1}) and dynamic viscoelasticity at a steady angular velocity (3.77 rad/s) were measured with a rheogoniometer equipped with a coaxial cylinder (18 mm diam.) and rotating outer cylinder (22 mm diam.), 60 mm long (IR-103, Iwamoto Seisakusho Co., Ltd., Kyoto). Circulating oil from a Thermo-cool (LCH-130F, Toyo Co., Ltd., Tokyo) controlled the temperature of the sample over the temperature range from 0 to 60°C, and raised it in steps at a rate of 1°C/min. Shear rate ($\dot{\gamma}$), shear stress (τ), and apparent viscosity (η) were calculated using the equation of Margules (Harris, 1977). Dynamic viscosity (η') and elasticity (G') were calculated by a modification of Markovitz's equation (Markovitz, 1952). The mechanical loss tangent was calculated from the relationship tan $\delta = G''/G'$ where G' is the loss modulus ($\omega\eta'$) and ω is the angular velocity of the outer cylinder.

Results

According to atomic absorption spectrophotometry, the cation concentration for the K, Na, and Ca salts of ι-carrageenan was 11.5, 7.2, and 6.2% (w/w), respectively. The native ι-carrageenan contained K^+, Na^+, and Ca^{2+} ions at a concentration of 2.2, 3.4 and 3.8% (w/w), respectively.

To compare the rheological behavior of aqueous solution of ι-carrageenan to that of commercial ι-carrageenan, viscosity and dynamic modulus were measured under the same conditions as
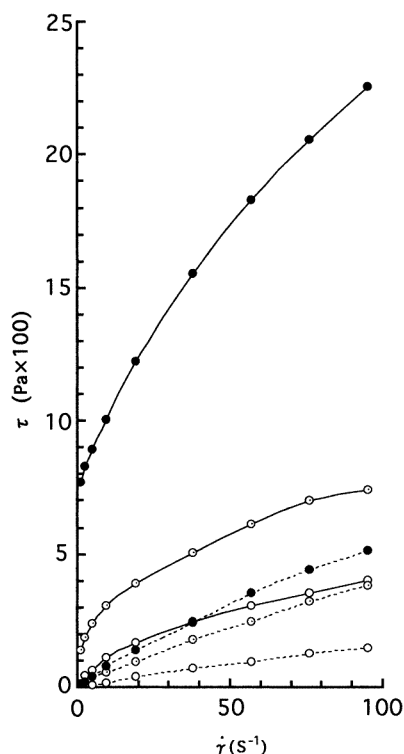
Fig. 2. Flow curves of an ι-carrageenan solution at various concentrations at 25°C. Full lines refer to the Ca-salt and broken lines to the K-salt. (●) 0.3% (w/v), (◉) 0.2% (w/v), (○) 0.1% (w/v).
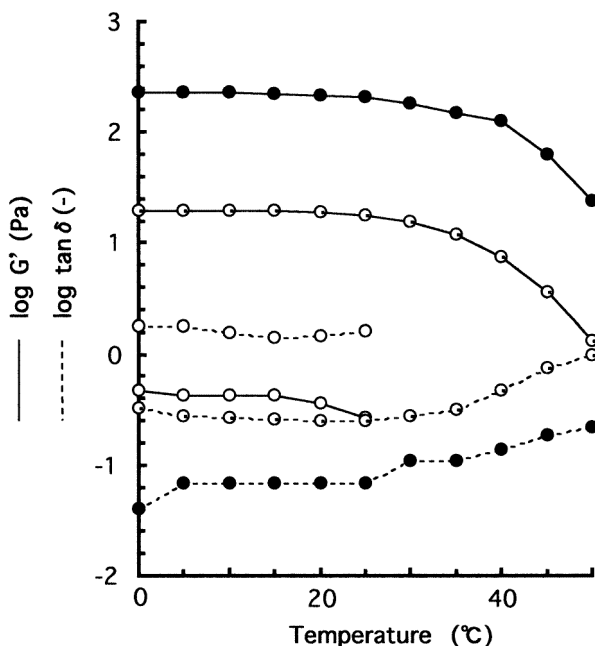

Fig. 3. Effects of temperature on the dynamic modulus, at 3.77 rad/s, of 0.1, 0.2 and 0.3% (w/v) solution of Ca-salt of ι-carrageenan at various concentrations. Full lines refer to the dynamic modulus and broken lines to the tan δ. (●) 0.3% (w/v), (◉) 0.2% (w/v), (○) 0.1% (w/v).

Fig. 4. Effects of temperature on the dynamic modulus, at 3.77 rad/s, of a 0.2% (w/v) solution of K- and Na-salt of ι-carrageenan a with addition of $CaCl_2$ (6.8 mM). Full lines refer to the dynamic modulus and broken lines to the tan δ. (●) K-salt only, (○) K-salt with $CaCl_2$, (◉) Na-salt only, (⊖) Na-salt with $CaCl_2$.

those of the previous study (Tako *et al.*, 1987). The flow curves, at 25°C, of K- and Ca- salts of ι-carrageenan at various concentrations are shown in Fig. 2. The flow curves for the Ca salt of ι-carrageenan showed plastic behavior and the yield stress was
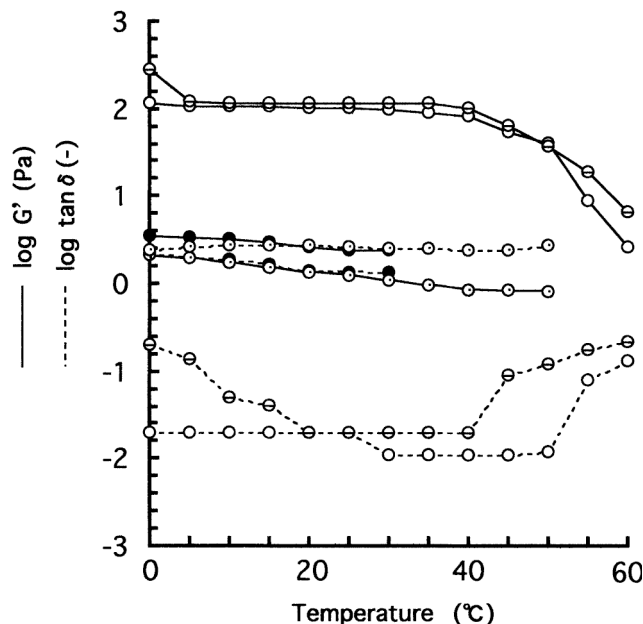
estimated to be 0.4, 1.7 and 7.7 Pa at 0.1, 0.2 and 0.3% (w/v) concentration, respectively. This indicates that an intermolecular association is stable at room temperature (25°C) (Tako *et al.*, 1987). The flow curves (Masson & Caines, 1954) of the Ca-salt of ι-carrageenan aqueous solution shifted to a higher shear-stress region than those of a commercial ι-carrageenan (Tako *et al.*, 1987), which showed shear-thinning behavior even just at the solution of 0.3% (w/v). However, the K-salt of ι-carrageenan aqueous solutions at a concentration below 0.2% (w/v) were approximated to Newtonian behavior, and to shear-thinning at a 0.3% (w/v).

Gelation occurred for the Ca salt of ι-carrageenan at concentrations >0.3% (w/v) at room temperature, but did not occur for the K- and Na- salt even at 2.0% (w/v) (not shown in Fig. 2), respectively. A small increase of the dynamic modulus was observed in the solution of K salt of ι-carrageenan, in contrast to that of the K salt of κ-carrageenan (Tako & Nakamura,1986; Qi *et al.*, 1997b). As shown in Fig. 3, the dynamic modulus of the Ca salt of ι-carrageenan (0.3% (w/v)) showed a very large value at low temperature (0°C) and remained at a constant value with increase in temperature up to 30°C. When the temperature reached 40°C, which was estimated to be a transition temperature, the dynamic modulus decreased rapidly.

A very large dynamic modulus was observed on addition of $CaCl_2$ (6.8 mM) for the Na and K salts of ι-carrageenan solution (Fig. 4). This suggests that the Na^+ and K^+ counter-ions were replaced with Ca^{2+} ions by addition of $CaCl_2$; consequently, a much greater intermolecular association took place (Tako *et al.*, 1987). The Na and K salts of ι-carrageenan in the presence of $CaCl_2$ showed lower values in tan δ than those of the Na and K salts of ι-carrageenan, indicating that a very strong association took place.
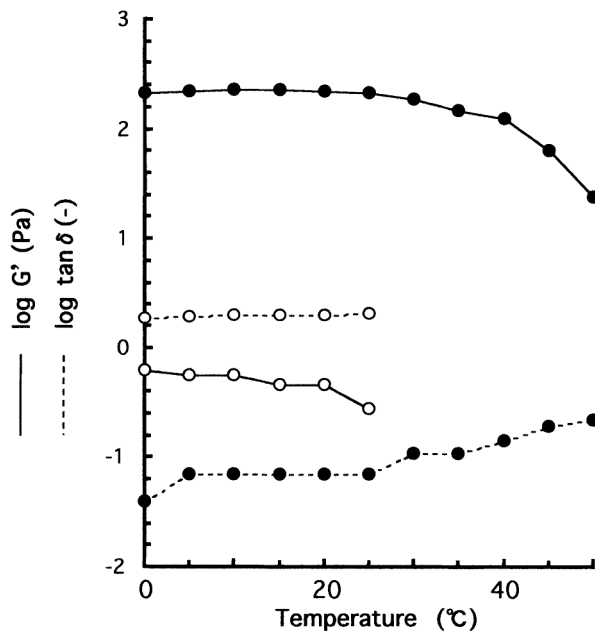
Fig. 5. Effects of temperature on the dynamic modulus, at 3.77 rad/s, of a 0.2% (w/v) solution of Ca-salt ι-carrageenan with addition of urea (4.0 M). Full lines refer to the dynamic modulus and broken lines refer to the tan δ. (●) Ca-salt ι-Carrageenan only, (○) with urea.
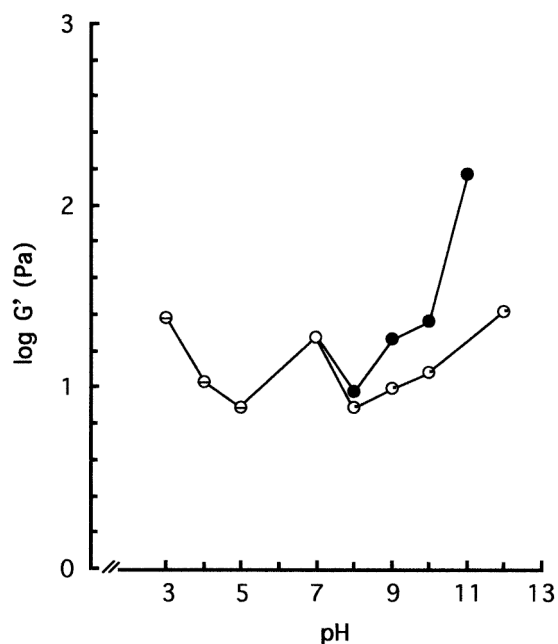

Fig. 6. Effects of pH on the dynamic modulus of Ca-salt ι-carrageenan at a concentration of 0.2% (w/v) at 25°C. The pH value was adjusted with 100 mM KOH (⊙), 100 mM Ca(OH)$_2$ (●), 100 mM HCl (⊖), and Ca-salt ι-Carrageenan only (○).

As shown in Fig. 5, the Ca-salt of ι-carrageenan was measured in the presence of urea (4.0 M), which is known as a hydrogen-bonding breaker, and showed a very small value in the dynamic modulus at a concentration of 0.2% (w/v). Thus, an ι-carrageenan association site seems to exist with hydrogen-bonding and urea prevents hydrogen-bonding among ι-carrageenan molecules.

As shown in Fig. 6, in the acidic pH range the dynamic modulus of Ca-salt of ι-carrageenan increased gradually and showed a maximum value at pH values of 3.05 by addition of 100 mM HCl. An increase of dynamic modulus was also observed in the alkaline range by addition of 100 mM Ca(OH)$_2$ and KOH at room temperature (25°C), and gelation occurred in that range after a pH of 11.03 and 12.04 was reached, respectively. The behavior in the alkaline range was parallel to that of commercial ι-carrageenan (Tako et al., 1987). This suggests that ι-carrageenan molecules associate tightly with Ca^{2+}, where sulfate group substituted on anhydro-D-galactose and D-galactose residues may contribute to cation bridges on different molecules with ionic bonding (Tako et al., 1987; Tako et al., 1989).

Discussion

The ι-carrageenan highly sulfated polysaccharides extracted from red seaweeds are widely used as functional ingredients in the food industry (Lynch & Mulvihill, 1996). The rheological data obtained provides information on the mechanisms by which the various cations modified the rheology of ι-carrageenan gels. The relative effects of K$^+$ and Ca^{2+} ions on the ordered conformation of ι-carrageenan were reported earlier (Bayley, 1955; Anderson et al., 1969; Arnott et al., 1974; Morris & Chilvers, 1981). The Ca^{2+} ions are more effective than the K$^+$ ions in promoting an intermolecular association of ι-carrageenan. These results were confirmed in a previous (Tako et al., 1987) and the

present report. Here, we measured the ι-carrageenan in both the viscosity and dynamic modulus under the same conditions, and found that that from Togekirinsai had higher gelling polysaccharide than that of commercial ι-carrageenan.

The Ca salt of ι-carrageenan from Togekirinsai showed a plastic behavior at lower concentration (0.1% (w/v)) than those of commercial ι-carrageenan and gelation occurred in 0.3% (w/v) at room temperature (25°C). The dynamic modulus of aqueous solution of Ca-salt of ι-carrageenan from Togekirinsai showed higher values than those of commercial ι-carrageenan. This can be attributed to the content of ester sulfate, anhydro-D-galactose and molecular mass (Masson & Caines, 1954; Anderson et al., 1973; Dawes, 1977; Yamada, 2000). The dynamic modulus of aqueous solutions of different cation forms of the ι-carrageenan extracted from Togekirinsai indicated the effects of the cations on gel-formation with divalent cations (such as Ca^{2+} ions) rather than monovalent cations (such as K$^+$ and Na$^+$ ions) (Arnott et al., 1974; Morris & Chilvers, 1981; Michel et al., 1997) under the experimental conditions described here. These results were consistent with the report concerning the relative effects of Ca^{2+}, K$^+$ and Na$^+$ ions on the ordered conformation of the commercial ι-carrageenan from Eucheuma spinosum (Tako et al., 1987). The transition temperature in the dynamic modulus was observed at 40°C. The ι-carrageenan molecule may adopt a single rod-like structure (Bayley, 1955) for the Na and K salts at room temperature. However, when the counter ions were replaced with Ca^{2+} ions upon addition of CaCl$_2$ and Ca(OH)$_2$, the intra- and inter-molecular Ca bridging on the different molecules could take place via ionic bonding and attractive electrostatic force (Tako et al., 1987).

These results support the widely accepted belief that ι-carrageenan is specifically gelled in the presence of divalent cations such as Ca^{2+} ions. Thus, gelation of the ι-carrageenan isolated

from Togekirinsai may take place in the manner shown in Fig. 1. According to the model, stabilization of the double-stranded helix should be held by the ionic bonding of attraction through Ca^{2+} ions at low temperature. In the double helical conformation, a tertiary structure of the ι-carrageenan molecules may consist of a three-fold right-handed helix as in the solid state (Bayley, 1955; Arnott *et al.*, 1974; Morris & Chilvers, 1981; Tako *et al.*, 1987).

In this study, ι-carrageenan isolated from Togekirinsai in the intra- and inter-molecular association mechanism through Ca bridges is the same as that of commercial ι-carrageenan, and shows a higher dynamic modulus at low concentration. This indicates that this ι-carrageenan is a highly gelling polysaccharide, which accounts for its being more extensively used than that of commercial ι-carrageenan. Thus, the rheological characteristics of ι-carrageenan isolated from Togekirinsai may be useful for further developments not only in industrial application, but also in understanding the biological functions.

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