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The Effect of Apparent Molecular Weight and Components of Agar on Gel Formation

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The relation between apparent molecular weight and physical properties of agar gel and sol were investigated. Agar was extracted from seven kinds of red seaweed, *Gelidium*, *Pterocladia* and *Gracilaria*, which were collected in different seas. The apparent weight-average molecular weight ($A\overline{Mw}$) and apparent molecular weight distribution of agar were measured by a high temperature type gel permeation chromatograph. Gel strength and melting point of agar gel, and the viscosity of agar sol were measured. The gel strength of agar gel from each seaweed increased with increasing $A\overline{Mw}$, and it was found that 3,6-anhydro-L-galactose and sulfate group affected gel formation. These results were also suggested by endothermic peaks of differential scanning calorimetry accompanying the gel-to-sol transition. The melting point of agar gel and the viscosity of its sol also increased with increasing $A\overline{Mw}$.

Keywords: agar, gel formation, apparent molecular weight

Agar is a polysaccharide which is extracted with hot water from a red seaweed such as *Gelidium* or *Gracilaria* species. It consists of agarose and agaropectin. Agarose is a linear polysaccharide composed of alternating D-galactose and 3,6-anhydro-L-galactose, while agaropectin is a polysaccharide containing sulfuric acid, D-glucuronic acid, pyruvic acid and so on, other than D-galactose and 3,6-anhydro-L-galactose (Araki, 1958). Agar is an important gelling agent used in the food industry as a texture modifier and in biotechnology as a bacterium medium, so the gelation mechanism and rheological and thermal behaviors of agarose and agar have been studied (Watase & Nishinari, 1987; Watase *et al*., 1990; Watase *et al*., 1992; Nishinari *et al*., 1992a). The physical properties of a polymer are believed to be influenced by its molecular weight, thus it is important for the agar industry to know the relation between the "molecular weight" of agar and the physical properties of a gel like its strength. A concept of molecular weight is used for a single molecule such as agarose or agaropectin; however, since agar is a mixture of these two molecules, the term "molecular weight" cannot be used. Let us define the "apparent molecular weight" of this mixture as an average of the molecular weight of agarose and the molecular weight of agaropectin as determined by gel permeation chromatography (GPC), which is often used to learn the molecular weight of a polymer. Agarose is thought to play a major role in the mechanical behavior of agar, and agaropectin also affects the behavior slightly. Although the relation between molecular weight and physical properties of agarose has been studied previously (Normand *et al*., 2000; Watase & Nishinari, 1983), there has been no report on agaropectin. From the viewpoint of industrial application, it is important to know the relation between the above-mentioned apparent molecular weight and physical properties. It is difficult both to prepare agar with different apparent molecular weight and to determine its apparent molecular weight. In the case of agar, since gelation of aqueous solution usually occurs at about 40 to 50°C, measurement of the apparent molecular weight by GPC must be performed at a higher temperature. But an ordinary GPC apparatus is only heated in a column compartment, making it difficult to make the measurement.

In a previous paper (Suzuki *et al*., 1999), we measured the apparent molecular weight of agar using a high temperature type GPC (HT-GPC) apparatus. This can be heated up to 150°C in its column, injector and detector compartments. Therefore, we do not need to take the gelation into consideration not only in a column but also in operating of sample injection. We then determined the optimum conditions for agar using the HT-GPC apparatus, and measured the apparent molecular weight distribution and apparent average molecular weight of agar which were prepared from two species of *Gelidium*. And the relation between the gel strength and the melting point of the agar gels and the apparent average molecular weight were almost linear, but the straight lines of the relation were not in agreement between agar gels extracted from two different *Gelidium* species.

In the present work, the relation was further investigated using agar gels extracted from several kinds of red seaweed, *Gelidium, Pterocladia* and *Gracilaria*. Effects of sulfate group and 3,6-anhydro-L-galactose in agar on gel formation were also examined, and the gel-to-sol transition of agar was studied by differential scanning calorimetry.

Materials and Methods

Materials The seven kinds of red seaweed collected in the different seas: *Pterocladia capillacea* from Portugal, *Gelidium sesquipedale* from the Azores (Portugal), *Gelidium linoides* from Ehime (Japan), *Gelidium amansii* from Kochi (Japan), *Gelidium amansii* from Izu (Japan), *Gelidium amansii* from Wakayama (Japan) and *Gracilaria chilensis* from Chile, were obtained from agar producing companies in Gifu prefecture (Japan). *Gracilaria chilensis* was pretreated in 5% sodium hydroxide aqueous solu-

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tion with occasional stirring at 85°C for 2 h. After this pretreatment, the seaweed was soaked in running water overnight to wash out excess sodium hydroxide, and air-dried at room temperature. The six kinds of *Gelidium* and *Pterocladia* were washed with water and air-dried for extraction without pretreatment. The various agars were extracted from these seaweed by the method described previously (Suzuki *et al*., 1999). Thirty-five grams of the air-dried seaweed was boiled in 1 *l* of water added with 1 N sulfuric acid for 1 h to extract agar. The volume of the added 1 N sulfuric acid was changed to prepare agars with different physical properties. The resulting mixtures were filtered, and the filtrates were placed in stainless trays and allowed to gel at room temperature. After gelation of each extract, the gel was cut into stringy form and frozen at –20°C overnight. The frozen gel was allowed to thaw. The process was repeated to dry. In this paper, agar extracted from the seaweed of Portugal, Azores, Ehime, Kochi, Izu, Wakayama and Chile are referred to as Portugal-Agar, Azores-Agar, Ehime-Agar, Kochi-Agar, Izu-Agar, Wakayama-Agar and Chile-Agar, respectively.

Chemical analysis The content of 3,6-anhydro-L-galactose in each agar was determined by the improved resorcinol method (Yaphe & Arsenault, 1965) using fructose as a standard material. Quantity of the sulfate group was determined by high performance liquid chromatography (ion chromatograph system, Showa Denko K.K., Tokyo; column, Shodex IC I-524A; eluent, 2.5 mM phthalic acid (pH 4.0); flow rate, 1.2 ml/min) from the trifluoroacetic acid (TFA) hydrolysate (Aoki & Miyazaki, 1998) of the agar (condition of hydrolysis, 50 mg of agar/5 ml 2N TFA, 1 h at 90°C). The quantity of uronic acid was determined by the carbazole-sulfuric acid method (Galambos, 1967) using glucuronic acid as a standard material. These contents were expressed as percentage to total carbohydrate which was measured by the phenol-sulfuric acid method.

Measurement of apparent molecular weight The determination of the apparent weight-average molecular weight ($A\overline{Mw}$) and of the apparent molecular weight distribution of agar was made by HT-GPC with a refractometer as a detector and an autosampler (Waters 150 CV GPC, Nihon Millipore Ltd., Tokyo) previously described (Suzuki *et al*., 1999). Two Shodex KS-805 (Showa Denko K.K.) columns were used and eluted with aqueous 0.2 M $NaNO_3$ at a flow rate of 0.6 ml/min. The column and injector compartments in the HT-GPC apparatus were thermostatted at 70°C. Samples were dissolved in the same solvent at 0.2% (w/v) by heating in a boiling water bath and were automatically injected (injection volume, 100 μl). A calibration curve was obtained by measuring pullulans (Shodex STANDARD P-82, Showa Denko K.K.) as standards.

Gel strength Gel strength was measured by the method commonly used for agar gel (Suzuki *et al*., 1999). Hot agar solutions dissolved in water at 1.5% (w/w) were poured into a stainless case (6 cm×20 cm×4 cm) and allowed to gel at 20°C overnight. The gel strength was measured using a Nikkansui measuring apparatus (Kiya Seisakusyo, Ltd., Tokyo), and was expressed as maximum load (N/m^2) below which the gel was not ruptured for 20 s.

Melting point Melting points were measured by the method of Hayashi & Nagata (1967). Hot agar solutions dissolved in water at 1.5% (w/w) were poured into test tubes (1.3 cmϕ×15 cm) and allowed to gel at 20°C overnight. The test tubes were inverted and allowed to stand in a water bath, which was then heated at the rate of 10°C/30 min. Melting point (°C) was expressed as the temperature at which the gel collapsed and a bubble occurred at the top of the test tube.

Viscosity Viscosity of 1.5% (w/w) agar solutions dissolved in water was measured by an EMILA rotary viscometer (Emila, Svendborg, Denmark) at 60°C. Shear rate was 1850 s^{-1}.

Differential scanning calorimetry (DSC) DSC measurements were carried out with a DSC120 (Seiko Instruments Inc., Chiba). A portion of 1.5% (w/w) agar gel (51.5±0.2 mg) kept at 20°C overnight was put into a 70 μl silver pan and heated at the rate of 2°C/min from 30°C.

Results and Discussion

There have been a few reports (Rochas & Lahaye, 1989; Murano *et al*., 1990; Murano *et al*., 1992) of chromatographic experiments for agar and agarose involving the gelling properties of these polysaccharides. Tako and Nakamura (1988) suggested that molecules of agarose in water may adopt a random coil above 60°C, and that the intra- and inter-molecular hydrogen-bonding may be formed at a temperature range from 60 to 40°C and below 40°C, respectively. The HT-GPC apparatus used in this study, all compartments of which were kept at a higher temperature, enables measurement of the apparent molecular weight of agar without inducing gelation.

Figures 1(a–c) show the relations between $A\overline{Mw}$ of agar extracted from various seaweed and the physical properties of the gel and sol. Table 1 shows the contents of 3,6-anhydro-L-galactose, sulfate group and uronic acid in various agar. The relations between gel strength and $A\overline{Mw}$ were found to be linear (Fig. 1(a)) as described previously (Suzuki *et al*., 1999). In agar extracted from the same seaweed, since the content of each of 3,6-anhydro-L-galactose, sulfate group and uronic acid was approximately the same (Table 1), gel strength was believed to be dependent on the molecular weight of agar as reported previously (Watase & Nishinari, 1983).

By comparing the relations between gel strength and $A\overline{Mw}$ of agar extracted from different seaweed, the linear relationships of

Table 1. Contents of agar extracted from various seaweeds.

Agar	$A\overline{Mw}$ ($\times10^5$)	3,6-anhydro-L-galactose (%)	SO_4^{2-} (%)	Uronic acid (%)
Chile-Agar	3.00	42.8	0.92	2.7
	1.73	43.0	1.0	2.8
	1.29	43.9	0.93	2.9
Portugal-Agar	2.40	40.6	0.98	3.8
	1.84	40.0	0.94	3.5
	1.27	39.4	0.96	3.7
Ehime-Agar	2.76	38.8	0.97	3.7
	2.46	39.8	0.91	3.4
	1.66	40.5	1.0	3.5
Kochi-Agar	3.49	37.9	1.7	2.8
	2.78	38.4	1.5	3.0
	2.30	39.0	1.7	3.1
Azores-Agar	3.89	39.4	2.7	2.4
	2.70	38.9	2.6	2.4
	2.29	40.5	2.9	2.4
Izu-Agar	3.01	36.3	1.1	3.3
	2.34	37.0	1.1	2.7
	1.49	37.8	1.1	3.0
Wakayama-Agar	4.19	36.3	1.3	4.1
	2.86	37.1	1.2	4.0
	1.73	36.5	1.3	3.5

Portugal-Agar and Ehime-Agar were found to be similar, and those of Kochi-Agar and Izu-Agar also to be similar. Except for these relations, the straight lines approximating the relation between gel strength and $A\overline{Mw}$ were found to be different. In other words, although $A\overline{Mw}$ of the agar extracted from each seaweed from the various seas was the same, the strengths of the gels differed. For example, Chile-Agar ($A\overline{Mw}$, 3.00×10^5; gel strength, 96 kN/m^2) and Wakayama-Agar ($A\overline{Mw}$, 2.86×10^5; gel strength, 37 kN/m^2) were approximately the same in $A\overline{Mw}$, while the gel strength (96 kN/m^2) of Chile-Agar was far larger than Wakayama-Agar (37 kN/m^2). The apparent molecular weight distribution of Chile-Agar (96 kN/m^2) shifted to slightly above that of Wakayama-Agar (37 kN/m^2) (Fig. 2), however, this did not seem to make a great difference in gel strength between them, because even Chile-Agar of which $A\overline{Mw}$ was 1.73×10^5 showed a gel strength of 64 kN/m^2. It has been reported (Watase, 1975; Watase & Nishinari, 1981a; Iida *et al*., 1988) that the gel formation was affected by the degree of regularity of alternation of D-galactose and 3,6-anhydro-L-galactose residues, and by content of the sulfate group. The gel forming power is governed by the existence of helices, and increase in degree of the regularity stabilizes the double helix structure of agar gel; the sulfate group in agar increases solubility of the agar in water. The content of 3,6-anhydro-L-galactose is usually used as an indication of the degree of regularity (Watase & Nishinari, 1981b; Watase & Nishinari, 1983). Position of the straight line which was approximated for the relation between $A\overline{Mw}$ and gel strength of Chile-Agar with the highest content of 3,6-anhydro-L-galactose is at the top of the graph (Fig. 1(a)). The position of other straight lines tended to be lower with decreasing content of 3,6-anhydro-L-galactose, and the straight line of Wakayama-Agar containing the lowest amount of 3,6-anhydro-L-galactose is at the bottom of the graph. That is to say, as compared with agar gels having the same $A\overline{Mw}$ extracted from different seaweed, the jelly strength tended to rise with increasing content of 3,6-anhydro-L-galactose.

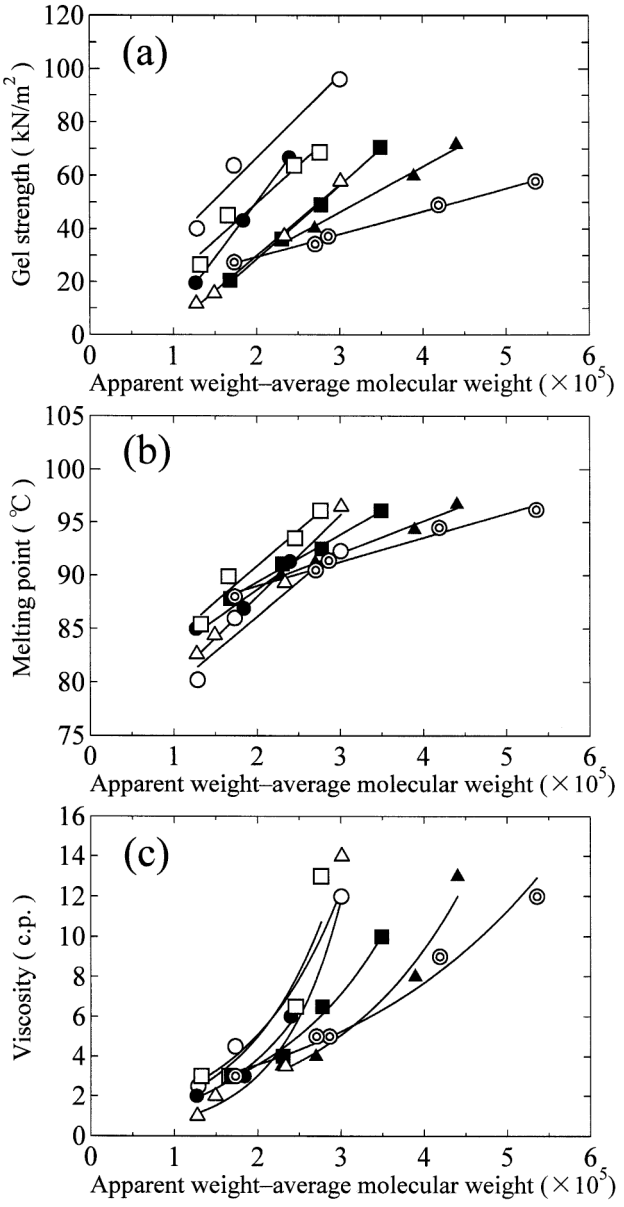

Fig. 1. Relations between apparent weight-average molecular weights and physical properties of gels and sols of Chile-Agar (○), Portugal-Agar (●), Ehime-Agar (□), Kochi-Agar (■), Azores-Agar (▲), Izu-Agar (△) and Wakayama-Agar (◎).

Watase *et al*. (1983, 1992) and Nishinari *et al*. (1987, 1992a, 1992b) investigated a gelation mechanism of aqueous solutions of polysaccharides by DSC. Endothermic peaks arising from the gel-to-sol transition as well as exothermic peaks arising from the sol-to-gel transition which were observed in heating and cooling DSC curves, respectively, were explained by the zipper model approach (Nishinari *et al*., 1990). It has been widely accepted that most thermo-reversible gels consist of somewhat crystalline regions, called junction zones, and somewhat amorphous regions. It is assumed that a junction zone consists of either an association of helices with secondary bonding such as hydrogen bonds, and that the number of junction zones, which is equivalent to the number of zippers in a zipper model approach, affects the gel formation. According to the model, DSC peak height is largely determined by the number of zippers.

Figures 3(a–c) show DSC curves of 1.5% (w/w) gels of Chile-Agar, Kochi-Agar and Wakayama-Agar. Chile-Agar ($A\overline{Mw}$, 3.00×10^5; gel strength, 96 kN/m^2), Kochi-Agar ($A\overline{Mw}$, 2.78×10^5;

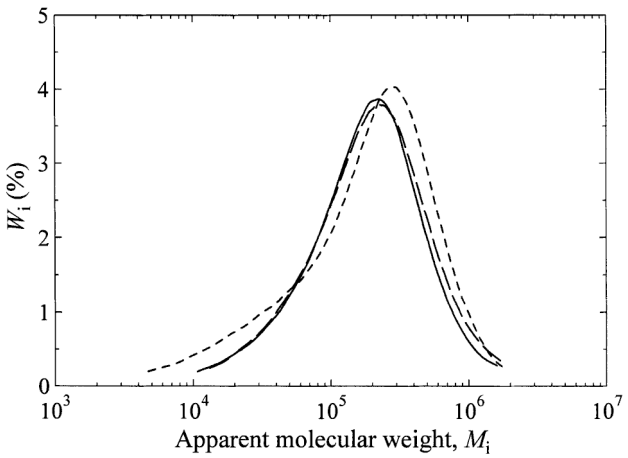

Fig. 2. Apparent molecular weight distribution curves from HT-GPC experiments of Chile-Agar ($A\overline{Mw}$, 3.00×10^5; ----), Kochi-Agar ($A\overline{Mw}$, 2.78×105; ——) and Wakayama-Agar ($A\overline{Mw}$, 2.86×10^5; — —). W_i is the per cent of polymer area of each apparent molecular weight, M_i on the chromatogram.

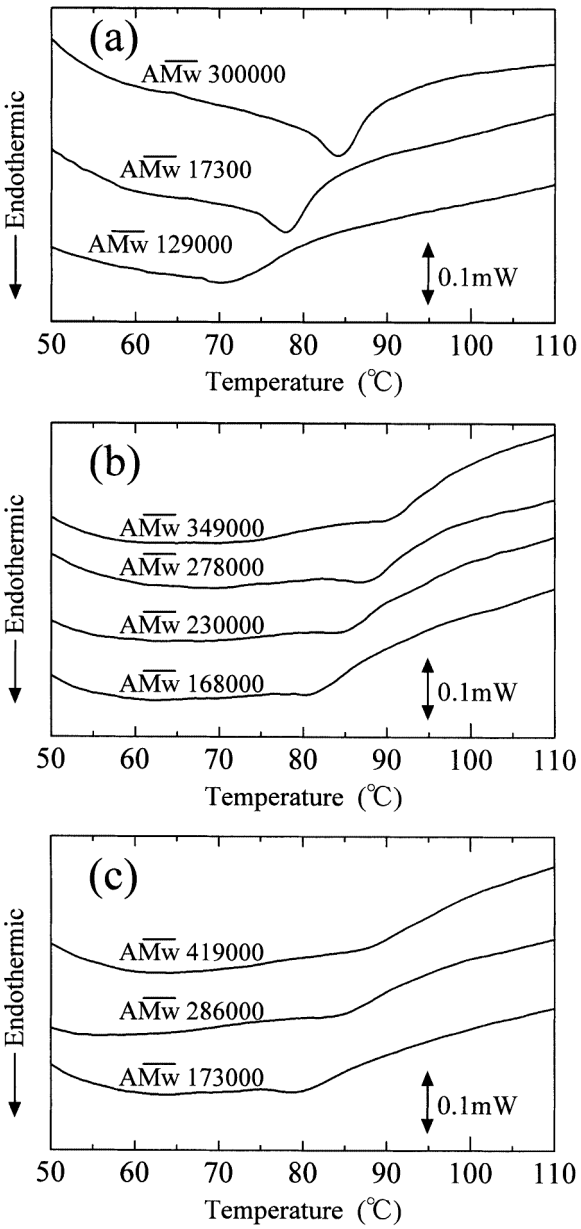

Fig. 3. DSC curve for gels of Chile-Agar (a), Kochi-Agar (b) and Wakayama-Agar (C) with various apparent weight-average molecular weights ($A\overline{Mw}$).

gel strength, 49 kN/m^2) and Wakayama-Agar ($A\overline{Mw}$, 2.86×10^5; gel strength, 37 kN/m^2) were approximately the same in $A\overline{Mw}$, and the apparent molecular weight distributions of these agars were not greatly different (Fig. 2). Endothermic peaks arising from the gel-to-sol transition were observed at 75 to 90°C in the DSC curves. Comparison of the DSC curves of these three kinds of agar showed the peak height of gel of Chile-Agar to be the highest and that of Wakayama-Agar the lowest. The zipper model approach was applied to these results, and the number of zippers or junction zones in the gel of Chile-Agar was the greatest and that of Wakayama-Agar the lowest; accordingly, the strength of the gel of Chile-Agar was the greatest and that of Wakayama-Agar the least.

The strength of agar gels extracted from the same seaweed primarily depended on the agar's $A\overline{Mw}$, and the relation between the gel strength and $A\overline{Mw}$ was almost linear. But there was no agreement of the straight lines for the relations of agar extracted from different seaweed, because each agar contained a different quantity of 3,6-anhydro-L-galactose. When the content of 3,6-anhydro-L-galactose was high such as in Chile-Agar, the components, sulfate group and uronic acid, which inhibit formation of the junction zones were believed to be lower, so that the strength of the gel was high because there were many of these zones. On the contrary, with low content of 3,6-anhydro-L-galactose such as in Wakayama-Agar, the gel strength was believed to be low because of the large quantity of components inhibiting junction zone formation. Although the content of 3,6-anhydro-L-galactose in Azores-Agar was slightly higher than Izu-Agar, position of the straight line of the former is lower in Fig. 1(a). The content of sulfate group in Azores-Agar was more than twice that of Izu-Agar. Therefore, the gel strength of agar gel was believed not only to depend on the content of 3,6-anhydro-L-galactose, but also to be influenced by the proportion of the components in the agar.

To confirm the above, multiple regression analysis (Kobayashi, 1981), which is a statistical analysis, was done with gel strength as a dependent variable, and $A\overline{Mw}$, and the contents of 3,6-anhydro-L-galactose, sulfate group and uronic acid as independent variables. The multiple correlation coefficient was 0.90, indicating they were strongly correlated. Their standardized partial regression coefficients were 0.96, 0.58, –0.57 and –0.24, respectively. These values statistically suggested the following. Gel strength is primarily influenced by $A\overline{Mw}$, and decreases with increasing contents of sulfate group and uronic acid, with the influence of former being greater.

In contrast to the DSC curves of agar with different $A\overline{Mw}$ extracted from each seaweed shown in Fig. 3, the endothermic peaks accompanying the gel-to-sol transition were shifted to higher temperatures with increasing $A\overline{Mw}$. This result was contrary to the methylcellulose gels described by Nishinari *et al.* (1997); they reported DSC curves of methylcellulose with various molecular weights. The endothermic peaks in heating DSC curves shifted to lower temperature with increasing molecular weight of methylcellulose. The reason is believed to be that the main bonding force in agar gel originates from hydrogen bonds, while hydrophobic interaction is responsible for the gel formation of methylcellulose.

The melting points shown in Fig. 1(b) are a rough indication of the temperature at the point of the gel-to-sol transition. The results for each seaweed showed that the melting points shifted to higher temperatures with increasing $A\overline{Mw}$ as did the endothermic peaks on DSC curves, and the relation between the melting points and $A\overline{Mw}$ was approximately linear. The angle of the slopes of the straight lines approximated for the relation of Azores-Agar and Wakayama-Agar was smaller than the others. It seemed that the high content of sulfate group in Azores-Agar and the low content of 3,6-anhydro-L-galactose in Wakayama-Agar affected the melting point.

The relation between $A\overline{Mw}$ and viscosity of agar solution of each seaweed is shown in Fig. 1(c). The viscosity increased with increasing $A\overline{Mw}$ as did the gel strength and melting point, but it seemed that the relation was roughly exponential rather than linear. Each exponential line was also different. In Azores-Agar ($A\overline{Mw}$, 3.89×10^5 and 4.40×10^5) and Wakayama-Agar ($A\overline{Mw}$,

4.19×10^5 and 5.35×10^5), the agar solutions were particularly lower in viscosity in spite of their high $\overline{AMw}$.

Since agarose plays a major role in the mechanical behavior of agar, it is urgent that the effect of molecular weight on the gel formation of agarose extracted from each seaweed be further studied.

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