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GELATION MECHANISM OF AGAROSE*

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ABSTRACT

The non-Newtonian behavior and dynamic viscoelasticity of a series of aqueous solutions of agarose were measured with a rheogoniometer. The flow curve, at 25°, of agarose solution approximated to plastic behavior at 0.1, 0.13, and 0.15% concentrations. Gelation occurred at concentration of 0.13% at low temperature (0°). The dynamic modulus of agarose showed a very high value at low temperature, and increased with an increase in temperature, showing a maximum value at 30°, then it decreased. In the presence of NaCl, KCl, $CaCl_2$, and $MgCl_2$ for a solution of agarose at 0.08% concentration, the transition temperature, at which dynamic modulus decreased rapidly, was observed at 60°. Gelation was also observed at low temperature (0°) in acid and alkaline range after reaching pH values of 2.3 and 9.5, respectively, by addition of 100mM HCl, H_2SO_4, NaOH, and $Ca(OH)_2$ to a 0.08% agarose solution. A possible mode of intra- and inter-molecular hydrogen bonding within and between the agarose molecules in aqueous solution is proposed.

INTRODUCTION

Agarose is the major gelling constituent of agar obtained from a family of red seaweeds *(Rhodophyceae)* and used in the separation, purification, and characterization of biomolecules in such techniques as gel filtration, affinity chromatography[1], and hydrophobic chromatography[2], as well as in the preparation of immunoadsorbants[3] and immobilized enzymes[4]. It consists of a copolymer alternating O-3-linked β-D-galactopyranosyl and O-4-linked 3,6-anhydro-α-L-galactopyranosyl residues[5,6], the structure of which is similar to that of *kappa*- and *iota*-carrageenan, except for the sulfate content and L configuration.

In the course of rheological studies of polysaccharides, we have discussed the molecular origin of their rheological characteristics[7–17], and proposed a gelation mechanism for κ- and ι-carrageenan in aqueous solution[15–17]. On the other hand, the physical properties of agarose have been extensively investigated[18–24], but the

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gelling mechanism of agarose is still not well understood at the molecular level.

In the present study, we analyze the rheological behavior of solutions of agarose with respect to its association characteristics in comparison with those of κ- and ι-carrageenan[15–17], and we propose a possible mode of intra- and inter-molecular hydrogen bonding in aqueous solution.

EXPERIMENTAL

Materials. — The sample of agarose was "Agarose-HGT(P)" (electrophoresis grade, Nakarai Co., Ltd.); it was a granular, white solid of M_r 120 000 containing 0.31% of ash and 0.15% of sulfate, and no D-glucuronic acid. The solutions were prepared in hot distilled water (95° for 20 min) at a concentration range of 0.08–0.15% (w/v).

Methods. — Specific rotation was measured at 589 nm with an automatic digital polarimeter DIP-180 (Japan Spectroscopic Co., Ltd.), for a solution of 0.1% (w/v) in water, with a cooling system. Viscosity at different shear rates (1.188–95.026 s^{-1}) and dynamic viscoelasticity at steady angular velocity (3.77 rad/s) were determined with a rheogoniometer consisting of a coaxial cylinder (1.8-cm diam.) with a rotating outer cylinder (2.2-cm diam.), 6.0-cm long (IR-103, Iwamoto Seisakusho Co., Ltd.). The temperature of the sample was controlled by circulating oil from a thermo-cool instrument (LCH-130F, Toyo Co., Ltd.), over the temperature range of 0–75° and raised at a stepwise rate of 1°/min. Shear rate (D), shear stress (S), and apparent viscosity (η) were calculated with the equation of Margules[25]. Dynamic viscosity (η') and elasticity (G') were calculated by a modification of Markovitz's equation[26]. The loss tangent was calculated from the relationship, $\tan \delta = G''/G'$, where $G'' = \omega\eta'$ is the loss tangent, and ω is the angular velocity of the outer cylinder.

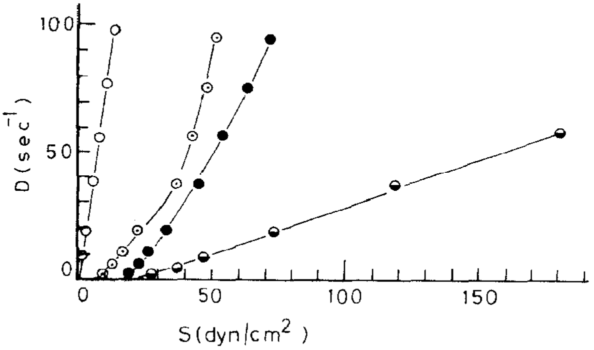


Fig. 1. Flow curves of agarose at various concentrations and 25°. Concentration: (○) 0.08, (⊙) 0.10, (●) 0.13, and (○) 0.15% (w/v).

RESULTS

The flow curve, at 25°, of agarose solution at various concentrations is shown in Fig. 1. For concentration of 0.08%, the flow curve approximated Newtonian, and for 0.1, 0.13, and 0.15% concentrations, plastic behavior. The yield value for the last-named concentrations were estimated to be 7, 18, and 23 dyn/cm^2, respectively. As reported previously[15], the flow curve of κ-carrageenan (native and potassium salt) showed Newtonian behavior at a concentration of 0.8%, pseudoplastic behavior at a concentration of 1.0%, and plastic behavior at concentrations of 1.5 and 2.0%, the yield values of the last-named were estimated to be 4 and 10 dyn/cm^2. The flow behavior, at 25°, of κ-carrageenan might be attributed to the dissociation of the intra- and inter-molecular K^+ bridges, which was caused by the increase of kinetic energy and Brownian motion of the polymer molecules and solvent[15]. On the other hand, the flow curve of ι-carrageenan (native and calcium salt) showed[12] plastic behavior at concentrations greater than 0.3%. This might be attributed to the formation of intra- and inter-molecular Ca^{2+} bridges, even at room temperature (25°). The flow characteristic of agarose differ from that of κ- and ι-carrageenan because it gelled even at a 0.2% concentration at room temperature; further, it showed plastic behavior even at a concentration of 0.1%, indicating that an even stronger association takes place between the agarose molecules than between the κ- or ι-carrageenan molecules.

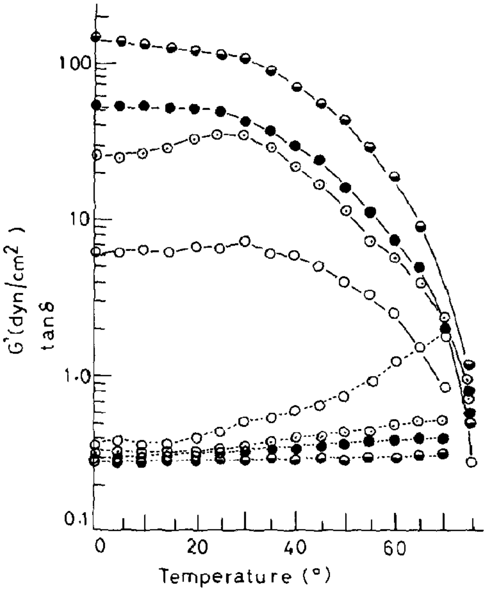


Fig. 2. Effects of temperature on the dynamic modulus at 3.77 rad/sec and various concentrations. Concentration: (○) 0.08, (⊙) 0.10, (●) 0.13, and (⊖) 0.15; (——) dynamic modulus and (------) tan δ.

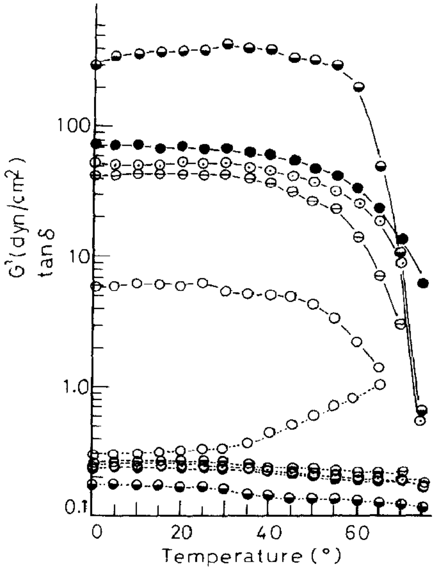


Fig. 3. Effects of temperature on the dynamic modulus, at 3.77 rad/sec, for a 0.08% solution of agarose (○) with addition of: 16.6mM NaCl (⊖), 13.5mM KOH (⊙), 6.8mM $CaCl_2$ (●), and 4.9 mM $MgCl_2$ (◓). The full lines refer to the dynamic modulus, and broken lines to the tan δ.

Gelation occurred at a concentration of 0.13% at low temperature, as shown in Fig. 2. The dynamic modulus of agarose at concentrations of 0.08 and 0.10% increased with increase of temperature, showing a maximum value at 30°, then it decreased, whereas for 0.13 and 0.15% concentrations, it stayed constant during increase of the temperature up to 30°, then it decreased gradually. The tan δ value was very low at ~0.2–0.3 for 0.10, 0.13, and 0.15% concentrations and stayed constant during increase of temperature up to 65°, then it increased. The tan δ of agarose at a 0.08% concentration showed a value of 0.3 at 0°, which stayed constant at temperatures up to 30°, then it increased gradually. This indicated that the intermolecular association of agarose molecules takes place at very low concentration.

As shown in Fig. 3, a very large increase of the dynamic modulus was observed, even at a concentration of 0.08%, in the presence of NaCl (16.6mM), KCl (13.5mM), $CaCl_2$ (6.8mM), and $MgCl_2$ (4.9mM) at low temperature (0°). The dynamic modulus in the presence of salt increased gradually with the increase of temperature, showing a maximum value at 30°, and then it decreased gradually. After the temperature reached 60°, which was estimated to be a transition temperature, the dynamic modulus decreased rapidly. The tan δ value of an agarose solution with salts was very low, ~0.18–0.25, at low temperature (0°), then it decreased gradually up to 75°. This result indicated that intermolecular association of agarose molecules is also stable for an increase in temperature in the presence of salts.

In the alkaline range, the dynamic modulus increased gradually and showed a maximum value at pH values of 9.5 and 9.7 by addition of 100mM NaOH and

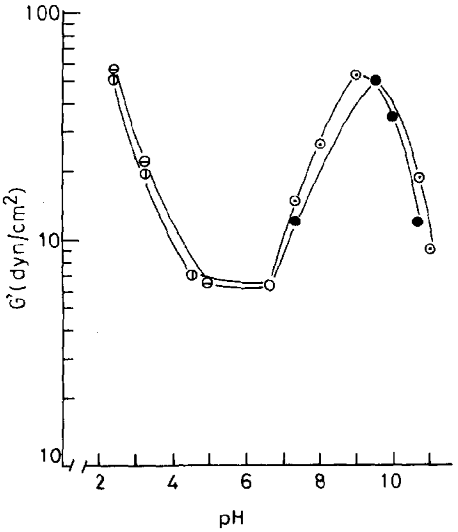


Fig. 4. Effect of pH on the dynamic modulus, at 3.77 rad/sec and the temperature of 0°, for a 0.08% solution of agarose (○) with addition of: 100mM HCl (⦶), H_2SO_4 (⊖), NaOH (⊙), and $Ca(OH)_2$ (●).

$Ca(OH)_2$ to a 0.08% agarose solution, and then it decreased rapidly (Fig. 4). An increase of dynamic modulus was also observed in the acid range by addition of 100mM HCl and H_2SO_4; after reaching a pH of 2.5, gelation occurred. The behavior in the acid range parallels that of κ- and ι-carrageenan, but differs in the alkaline range[15,16], which may be due to the non-ionic character of the agarose molecule.

The dynamic viscoelasticity of agarose in solution (0.08%) was measured in the presence of urea (4.0M), which is known as a hydrogen-bonding breaker. The least dynamic modulus was observed, indicating that urea prevents hydrogen-bonding in agarose molecules.

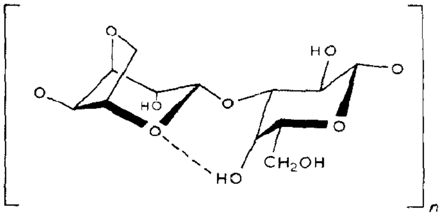
The specific rotation of a 0.1% solution of agarose at various temperatures was determined after dissolving the sample at 95°, and then cooling the temperature from 80° to 20° (Table I). The specific rotation increased with decreasing temperature up to 60°, where it stayed constant at $[\alpha]_D$ −30°; after the temperature reached 40°, the specific rotation increased gradually with a further decrease of the temperature. These data indicated that a two-steps mechanism exists for a conformational transition[27] at the temperatures of 60° and 40°, respectively.

TABLE I

SPECIFIC ROTATION OF AGAROSE[a]

Temperature (degrees)	80	70	60	50	40	30	20
$[\alpha]_{589}$ (degrees)	−27	−28	−30	−30	−30	−37	−38

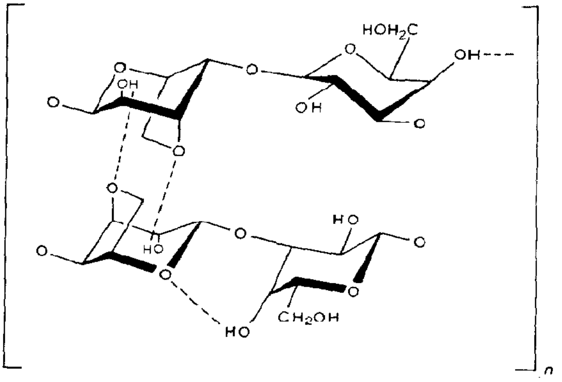
[a] For a solution in water, c 0.1%.



Scheme 1. Possible model of intramolecular hydrogen-bonding of agarose in aqueous solution. The dotted line refers to the hydrogen bonding.

DISCUSSION

The changes of the dynamic modulus of an agarose solution are in agreement with the specific rotations, suggesting an intramolecular association[11,15,17]. Consequently, we propose a mode of intramolecular hydrogen-bonding between OH-4 of the D-galactopyranosyl residue and the adjacent hemiacetal oxygen atom of the anhydro-α-L-galactopyranosyl residue (Scheme 1), below the transition temperature (60°). This bonding is likely owing to the flexibility of the α-L-(1→3) linkage between the anhydro-L-galactosyl and D-galactosyl residues. The anhydro-α-L-galactosyl residue is a cage-like residue that contributes to the rigidity of the molecular chain of agarose, and stabilizes the proposed intramolecular hydrogen-bonding. In addition, an intermolecular hydrogen-bonding may be proposed between the ring O-3,6 atom and OH-2, which has an axial orientation and is bound with less flexibility (Scheme 2). This intermolecular bonding results from the cage effect of the anhydro-α-L-galactosyl residue, which may adopt a tetrahedral distribution and, therefore, may attract not only each other, but also water molecules with hydrogen bonding. The intermolecular hydrogen bonding may exist below a temperature of 40°. The result of the specific rotation determination suggested that the molecule of agarose may adopt a random coil >60°, but that it may keep a rod-like conformation at the temperatures ranging from 60 to 40° owing to the formation of intra-



Scheme 2. Possible model of intermolecular hydrogen-bonding of agarose molecules in aqueous solution. The dotted lines refer to the hydrogen bonding.

molecular hydrogen-bonding. Consequently, the intermolecular hydrogen-bonding may take place at a temperature <40°, even for a low concentration.

The intra- and inter-molecular hydrogen-bondings result in gelation up to a high temperature (60°) and insolubility in cold water, because, as the polymer molecules bind tightly, their periphery is surrounded by hydrophobic carbon and hydrogen atoms. The agarose molecules are hydrated at a high temperature >90°, where dissociation occurs owing to an increase of kinetic energy and the Brownian motion of polymer and water molecules.

A large increase of the dynamic modulus was observed on addition of salts (NaCl, KCl, $CaCl_2$, and $MgCl_2$) and in the alkaline range (showing a maximum value at pH 9.5–9.7). This may be caused by a salting-out effect, which may take place because of the rigidity of the molecular chains of agarose through the formation of intra- and inter-molecular hydrogen-bondings, resulting from the tetrahedral distribution of anhydro-α-L-galactosyl residues.

This model corresponds to a double-stranded helix. A tertiary structure of agarose molecules may consist of two identical, left-handed, three-fold helices in aqueous solution as in the solid state[28]. Consequently, we suggest that the cavity in the double-stranded helix is occupied by water molecules, the arrangement of which are very nearly tetrahedral, in a manner similar to that of the anhydro-α-L-galactosyl residues in the aqueous solution.

Although agarose is a diastereomeric derivative of κ- or ι-carrageenan having a smaller sulfate content, it shows an intramolecular association similar to that of the carrageenans[15,17]. The role of such polysaccharides in biological tissues seems to be the cohesion, the retention of water and salts, and the physical organization. Thus, the gelation mechanisms for these polysaccharides, proposed in this and previous[15,17] studies, may be useful for further developments of not only industrial applications, but also for an understanding of the biological functions.

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