
Structure-Function Relationship of Rhamnan Sulfate Isolated from Commercially Cultured Edible Green Seaweed, *Monostroma nitidum*

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Abstract: The green seaweed, *Monostroma nitidum*, is widespread in Japan. In Okinawa Prefecture, production of seaweed is performed using culture-nets that are seeded artificially. The annual production of the algae in Okinawa was approximately 100t in 2016. Recently, because *Monostroma nitidum* is used in salads, soups and other items, its utilization in the food industry increased. The algae contain a soluble polysaccharide, rhamnan sulfate. To estimate the applicability of a rhamnan sulfate as a food additive or non-food additives, we investigated the rheological properties of the polymer that was isolated from commercially cultured *Monostroma nitidum* using a rheogoniometer. A soft gelation occurred at a concentration of 4.0%, and the elastic modulus stayed at a constant value after the temperature to 50°C, which was estimated to be a transition temperature, then decreased rapidly with further increase in temperature. Although a small decrease in elastic modulus was observed with the addition of urea (4.0 M), it remained constant with an increase in temperature up to 60°C, and then decreased. An increase in the elastic modulus was observed in a 0.05 M NaOH solution and soft gelation occurred. The elastic modulus remained large during the increase in temperature even at 90°C. A soft gelation also occurred when rhamnan sulfate was dissolved in a Tris buffer (pH 8.0) solution. The possible mode of intra- and intermolecular associations within and between rhamnan sulfate molecules were discussed.

Keywords: Green Seaweed, *Monostroma nitidum*, Rhamnan Sulfate, Structure-Function Relationship, Intra- and Intermolecular Associations, Gelation Mechanism

1. Introduction

Polysaccharides derived from microorganisms, plants and animals are used as thickening, stabilizing and gelling agents. Each polysaccharide has special properties that result from its individual molecular structure. We discussed the structure-function relationship from the view point of rheological characteristics of the polysaccharides and proposed the gelation mechanism of κ -carrageenan [1-3], ι -carrageenan [4, 5], agarose (agar) [6] gellan gum [7], amylose [8], curdlan [9], alginic acid [10, 11], rice starch [12-14], potato starch [15], wheat starch [16, 17], deacetylated rhamnan gum [18] and native gellan gum [19] at the molecular

level in aqueous solutions. The hydroxyl, hemiacetal and/or methyl groups of sugar residues participated in intra- and/or intermolecular associations with hydrogen bonding and van der Waals forces of attraction in neutral polysaccharides solutions, such as agarose, amylose, curdlan, gellan gum, deacetylated rhamnan gum and starches. The sulfate and carboxyl groups of the sugar residues of some acidic polysaccharides, such as κ -carrageenan, ι -carrageenan, gellan gum, alginic acid, deacetylated rhamnan gum and native gellan gum, might also participate in intra- and/or intermolecular associations through univalent or divalent

cations with ionic bonding or electrostatic forces of attraction.

We also proposed a co-gelation mechanism between xanthan and galactomannan (locust-bean gum [20-23], guar gum [24], tara-bean gum [25], Leucaena gum [26], and Leonix gum [27]), and between xanthan and glucomannan [28].

Furthermore, we proposed intramolecular associations for the gellan family of polysaccharides, welan [29], rhamsan [30], S-657 [31], S-88 [32] and S-198 [33] gum where methyl groups of the L-rhamnosyl residue plays an important role, leading to their thermal stability in aqueous solutions.

Consequently, we realized, in principle, that there are some basic rules for in gel-formation processes including water molecules and polysaccharides at the molecular level [34, 35]. The gelation occurs in the formation of intramolecular association to make polymer molecule rigid, then intermolecular associations take place in aqueous solutions. We also reported the principle of amylose, amylopectin and starch gelatinization as well as retrogradation including water molecules at the molecular level [36].

Some edible green seaweeds as well as brown and red ones are grown in Okinawa Islands. We isolated rhamnan sulfate (Ulvan) from an edible green seaweed, *Ulva pertusa*, and proposed chemical structure (heptasaccharide repeating units) as shown in Figure 1 [37] which was revised a little because oligosaccharides, β -D-glucuronic acid-(1 \rightarrow 4)- α -L-rhamnopyranose-(1 \rightarrow and α -L-idulonic acid-(1 \rightarrow 4)- α -L-rhamnopyranose-(1 \rightarrow were identified in published papers [38-40]. The ulvan consisted of 1,4-linked α -L-rhamnopyranosyl (3 molar) and D-xylopyranosyl (1 mol.), and 1,3-linked α -L-rhamnopyranosyl (1 mol.) residues on the main chains. Terminal D-glucuronopyranosyl (1.5 mol.) and L-idulonopyranosyl (0.5 mol.) residues were attached at C-2 and/or C-4 of L-rhamnosyl residues on the main chains. The sulfate groups were attached at C-2 and C-3 of the L-rhamnopyranosyl residue as well as C-3 of the D-xylopyranosyl residues. Ulvan is known to exhibit gel [39, 40].

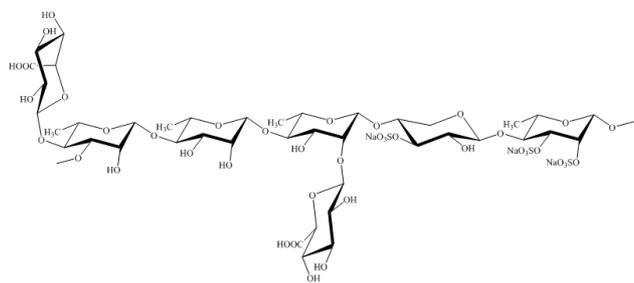


Figure 1. Chemical structure of rhamnan sulfate (Ulvan) isolated from *Ulva pertusa*.

The green seaweed, *Monostroma nitidum*, is widespread in nature between Korea, Japan and China. In Okinawa Prefecture, Japan, the production of the seaweed is performed using culture-nets that are seeded artificially. The annual production of the alga in Okinawa was approximately 100 t in 2016. Recently, because *M. nitidum* is used in salads, soups and some other items, its utilization in the food industry has

increased. We reported the chemical structure of rhamnan sulfate (octa-saccharide repeating units) that was isolated from commercially cultured an edible green seaweed, *M. nitidum* [41]. The rhamnan sulfate consists of 1,3-linked α -L-rhamnopyranosyl residues on the main chain, a part of which

has β -D-glucuro-pyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranosyl trisaccharide side-chains at the C-2 position on the main-chain. The sulfate groups are substituted at the C-4 position of the L-rhamnopyranosyl residues on the main-chain, and at C-3 position on the side-chains, as shown in Figure 2.

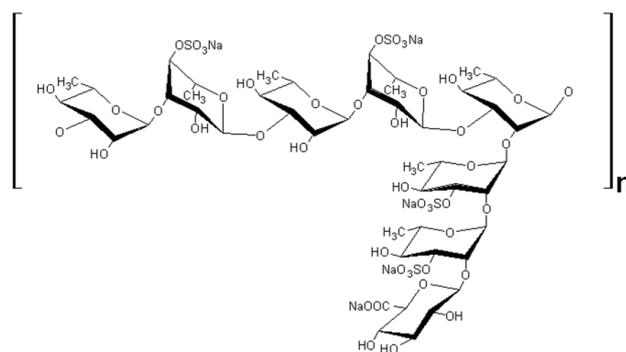


Figure 2. Chemical structure of rhamnan sulfate isolated from *Monostroma nitidum*.

To estimate the applicability of rhamnan sulfate as food additives or non-food additives, we report the rheological characteristics of the polysaccharide. Its structure-function relationship was analyzed with respect to the association characteristics in aqueous solutions.

2. Materials and Methods

2.1. Materials

Monostroma nitidum, which was commercially cultured on nets (1.5 \times 20 m) at sea off the central part of Okinawa Island (Kitanakagusuku Village), was used in this study.

2.2. Preparation of Rhamnan Sulfate

An air-dried seaweed sample (20 g) was suspended in 0.05 M HCl and stirred at 60°C for 3 h to extract rhamnan sulfate. The extract was then centrifuged at 13,000g for 20 min, and the supernatant was filtered through Celite 545. The filtrate was neutralized with 0.5 M NaOH and then precipitated by the addition of 2 volumes of ethanol. The precipitate was dried *in vacuo* [41].

The crude polysaccharide was dissolved in distilled water and the solution was passed through Celite 545. The filtrate was deionized by passing through a cation exchange column composed of Amberlite 120A H⁺ (Organo, Japan). The rhamnan sulfate solution was neutralized with a 0.05 M NaOH solution. The solution was subsequently precipitated by the addition of 2 volumes of ethanol. The precipitate was dried *in vacuo* [41].

2.3. Determination of Total Carbohydrate, Uronic Acid and Sulfuric Acid of Rhamnan Sulfate

The composition of the total carbohydrate and uronic acid was determined by the phenol-sulfuric acid method [42] and the carbazole-sulfuric acid method [43] using L-rhamnose and D-glucuronic acid as standards, respectively. Sulfuric acid was determined by the turbid-metric method described by Dodgson and Price [44].

2.4. Molecular Mass Determination of Rhamnan Sulfate

The molecular mass of rhamnan sulfate (1 mg/mL) was determined by high-performance liquid chromatography (HPLC), using an LC-6A chromatograph (Shimadzu, Kyoto Japan) on a TSK-gel GMPW column (7.8×200 mm, Tosoh Corporation, Tokyo, Japan). The HPLC analysis was performed at a flow rate of 0.3 mL/min with refractive index detection (RID-6A, Shimadzu) at room temperature. The column was conditioned with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2), and elution was conducted with the same buffer. Standard pullulans (Showa Denko Co. Ltd., Tokyo, Japan) including P-800 (molecular mass, 810kDa), P-400 (400kDa), P-200 (220kDa), P-100(110kDa) and P-80 (80kDa) were used as molecular mass markers.

2.5. Rheological Measurements

An aqueous solution was obtained by stirring rhamnan sulfate for 30 min at room temperature. The viscosity at various shear rates (1.19-95.03 s⁻¹) and the dynamic viscoelasticity at a fixed frequency (3.77 rad s⁻¹) were determined with a rheogoniometer (Iwamoto Seisakusho Co., Ltd, Japan) consisting of a coaxial cylinder (1.8 cm diam.) with a rotating outer cylinder (2.2 cm diam.). The temperature of the sample was controlled by circulating oil from a thermo-cool instrument (LCH-130F, Toyo Co., Ltd, Japan), over the temperature range of 0 to 90°C that was increased at a stepwise rate of 1°C min⁻¹. Shear rate ($\dot{\gamma}$), shear stress (τ), and viscosity (η) were calculated with the equation of Margules [45]. The dynamic viscosity (η') and elastic modulus (G') were calculated by modification of Markovitz's equation [46]. The loss tangent ($\tan \delta$) was calculated from the relationship, $\tan \delta = G''/G'$, where $G'' = \omega \eta'$ is the loss modulus, and ω is the angular velocity of the outer cylinder.

3. Results

3.1. Chemical Characteristics and Molecular Mass of Rhamnan Sulfate

The yield of rhamnan sulfate from commercially cultured *M. nitidum* was 21.7% and 5.4% (W/W) on the basis of dried and wet algae. The total carbohydrate, D-glucuronic acid and sulfate contents of the rhamnan sulfate were estimated as 64.1%, 13.5% and 21.2%, respectively. The molecular mass of rhamnan sulfate was 600kDa.

3.2. Flow Properties

The flow curve at a concentration of 2.0% approximated shear-thinning behavior and increased gradually with increase in shear force (not shown in Figure). The flow curve of the 3.0 % solution showed plastic behavior, the yield value of which was estimated to be 0.5 Pa, which increased gradually with an increase in shear force. For the 4.0% solution, the flow curve also showed plastic behavior, the yield value of which was 0.8 Pa, and increased linearly up to 9.5 s, then increased gradually. The yield value indicates that a secondary association (intra-and/or intermolecular) is involved in rhamnan sulfate molecules at high concentrations (3 and 4%) in aqueous solution [1-19].

Logarithmic plots of shear rate and shear stress for the rhamnan sulfate, as shown in Figure 3, showed a refraction point at concentration of 2, 3, and 4%. Such refraction point was also observed for locust-bean gum (single side chain) [47], guar gum (single side chain) [47] and xanthan gum (trisaccharide side chain) [48] where side chains of those polysaccharides contributed to entanglements of the molecular chains under shearing force in aqueous solutions. The results suggested that the rhamnan sulfate involved side chains as in locust bean gum, guar gum and xanthan gum. The result supports the chemical structure of the rhamnan sulfate (Figure 2) as reported by the authors [41].

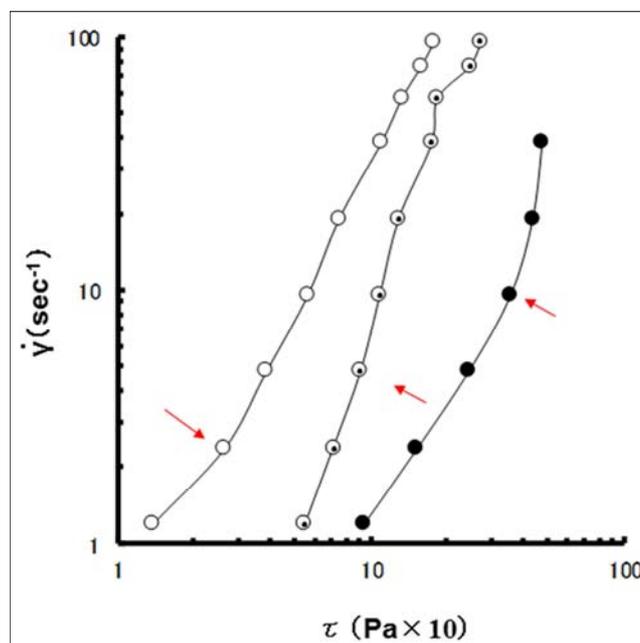


Figure 3. Logarithmic plot of flow curves of rhamnan sulfate from *M. nitidum* at various concentrations at 25°C.

→, Refraction point
Concentration: ○, 2% ; ⊙, 3% ; ●, 4%.

3.3. Elastic Modulus

The elastic modulus of rhamnan sulfate decreased gradually with increasing temperature to 30°C, which was estimated to be a transition temperature, however, it stayed

constant value with further increase in temperature up to 45°C, and then decreased in the 2.0% solution (not shown in Figure). A large elastic modulus was observed in the 3.0% solution at low temperature (0°C) and remained constant with increasing temperature to 45°C, which was estimated to be a transition temperature, and then decreased rapidly. A very large elastic modulus was observed in the 4.0% solution and gelation occurred at low temperature (0°C). The elastic modulus remained constant with increasing temperature to 45°C, which was estimated to be a transition temperature, and then decreased rapidly. On the other hand, the $\tan \delta$ value was large (1.2) at a low temperature (0°C), increased gradually with increasing temperature to 15°C, and then remained constant. After reaching 50°C, the value slightly decreased in the 2.0% solution. The $\tan \delta$ decreased to 0.36 with increasing concentration to 3.0% at low temperature (0°C) and remained constant with increasing temperature up to 40°C, which was estimated to be a transition temperature, then slightly decreased. A smaller $\tan \delta$ was observed when the concentration increased to 4.0% (0.25). The value remained constant up to 45°C, which was estimated to be a transition temperature, and then increased. The transition temperature is the temperature above which the secondary association dissociates. The results indicate that rhamnan sulfate molecules have a thermostable characteristic in aqueous solution as in gellan family of polysaccharides [29-33].

As shown in Figure 4, a small decrease in the elastic modulus was observed upon addition of 4.0 M urea [6, 8, 9] to the 4.0% aqueous solution of rhamnan sulfate, but the elastic modulus remained constant with increasing temperature to 60°C, which was estimated to be a transition temperature, and then decreased rapidly. The $\tan \delta$ shifted higher than in aqueous solution (0.25) upon addition of urea (0.37) at low temperature (0°C) and increased with increasing temperature to 10°C, but stayed constant until 60°C, which was estimated to be a transition temperature. The $\tan \delta$ then increased with further increases in temperature. A small decrease in the elastic modulus at low temperature (0°C) indicates that hydrogen bonding is involved in rhamnan sulfate molecules in aqueous solution and indicated that another secondary association was involved because the elastic modulus was large and remained constant up to the transition temperature (60°C) in a 4.0 M urea solution. Another secondary association dissociated rapidly above the transition temperature. The result suggests that van der Waals forces are co-involved in addition to hydrogen bonding [7, 8, 19, 29].

A very large elastic modulus approximately 10 times that of the aqueous solution (4.0%) was observed when rhamnan sulfate was dissolved in 0.05 M NaOH solution [12-17] at a low temperature (0°C) and soft-gel occurred as shown in Figure 5. The elastic modulus remained large during the increase in temperature even at 90°C. However, $\tan \delta$ value was large even at low temperature (1.0), slightly increased with increasing temperature to 80°C, and then decreased. The result indicates that soft gelation takes place even after becoming free from hydrogen bonding, because NaOH is also a hydrogen bonding breaker [12-17]. The result suggests that

van der Waals forces of attraction was involved in the intra- and intermolecular association in aqueous solution. The increase in the $\tan \delta$ value may be caused by association between trisaccharide side-chains on different molecules as in Ca-salt of xanthan molecules [49]. The result suggests that Na^+ bridges between sulfate groups of long side chains were built up with ionic bonding and electrostatic forces of attraction at high concentration in alkaline solution.

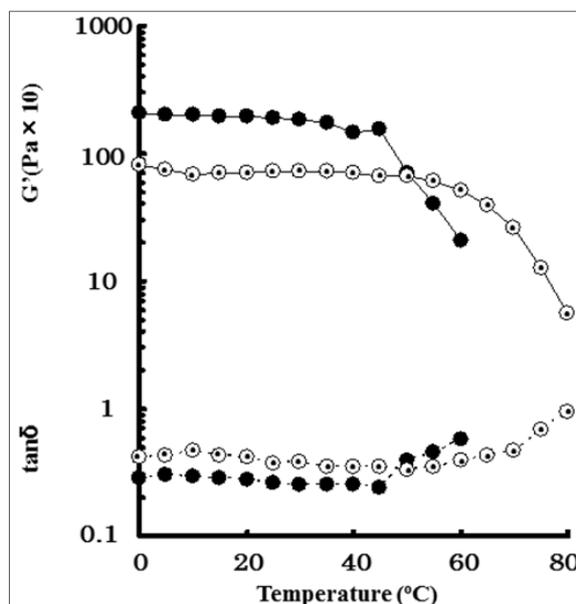


Figure 4. Effect of temperature on the elastic modulus of rhamnan sulfate (concentration, 4.0%; ●) from *M. nitidum* with addition of 4.0M urea (○).

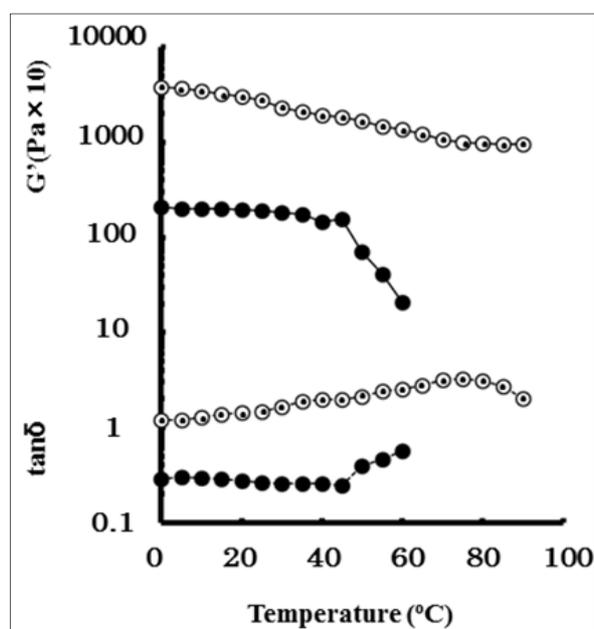


Figure 5. Effect of temperature on the elastic modulus of rhamnan sulfate (4.0%, ●) from *M. nitidum* in alkaline solution (0.05M-NaOH, ○).

Figure 6 shows the effect of temperature on the elastic modulus and $\tan \delta$ of the rhamnan sulfate at 3.0% in Tris-HCl (10 and 20 mM) buffer (pH 8.0) solutions. Very large elastic moduli, approximately 20-30 times of those of aqueous

solution, were observed in both buffer solutions, and soft gelation occurred. The elastic moduli were constant with increase in temperature up to 60°C, which was estimated to be a transition temperature, and then decreased rapidly. The $\tan \delta$ was larger, 1.0 and 1.6, in 10 and 20 mM buffer solutions, than in aqueous solution (0.36) at low temperature (0°C), increased with increasing temperature up to 45°C, and then decreased with further increases in temperature. The result suggests that rhamnan sulfate molecules associate with 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris) with ionic bonding and electrostatic forces of attraction between the side-chains of the sulfate groups on the different molecules of the rhamnan sulfate.

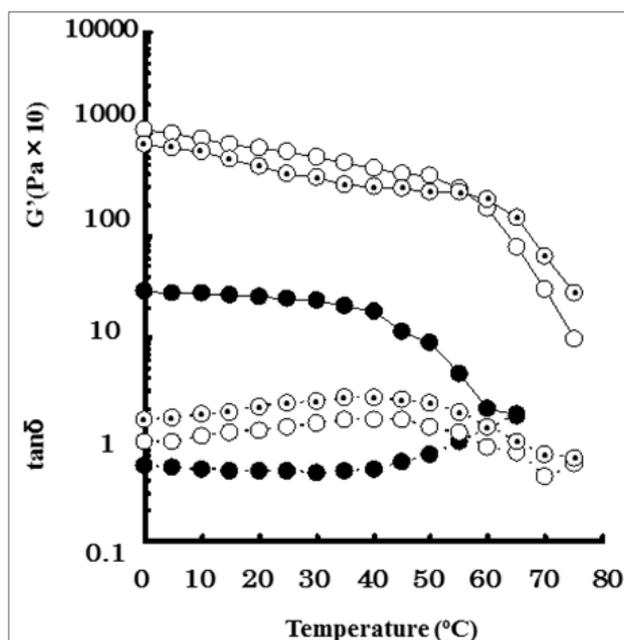


Figure 6. Effect of temperature on the elastic modulus of rhamnan sulfate from *M. nitidum* (3%; ●) in Tris-HCl buffer (10mM; ○ and 20mM; ○, pH8.0).

4. Discussion

The L-rhamnosyl residue plays a dominant role in gel-formation and thermostable processes in native [19] and deacylated [7] gellan gum, and native [30] and deacetylated rhamnan [18] gum solutions. Although the methyl groups of the L-rhamnosyl residues co-exist in 1C_4 and 4C_1 pyranose-ring conformations in native gellan and rhamnan gum molecules, resulting in soft-gel formation or thermal stability, they showed stiff and brittle gels on deacylation or deacetylation by treatment with a 0.2 M NaOH solution, where almost all of the L-rhamnosyl residues existed the 4C_1 pyranose-ring conformation [7, 18, 19]. Such a flexible conformational change might also occur in the rhamnan sulfate molecules in an alkaline solution.

A soft gelation occurred at low temperature (0°C) at a concentration of 4.0% in the rhamnan sulfate aqueous solution. Thus, we proposed possible mode of intra- and intermolecular associations. Intramolecular hydrogen bonding may take

place between the OH-4 of the L-rhamnosyl residue which may adopt the 1C_4 pyranose-ring conformation and adjacent hemiacetal oxygen atom of the L-rhamnosyl residue, which may adopt the 4C_1 pyranose-ring conformation within molecules to make them rigid. An intermolecular van der Waals forces of attraction may take place between the methyl group adopting the axial orientation of the L-rhamnosyl residue and the hemiacetal oxygen atom of the L-rhamnosyl residue (on the main-chain and/or side-chain) on different molecules as in deacylated gellan [7], deacetylated rhamnan [18] and native gellan [19] gum molecules.

A soft gelation also occurred in alkaline (0.05 M NaOH) solution, even after becoming free from intramolecular hydrogen bonding which may be caused by a conformational change, 1C_4 pyranose-ring to 4C_1 pyranose-ring conformational change on the L-rhamnosyl residues [18,19], where the OH-4 and methyl (C-6) groups adopted the axial orientation. Consequently, intermolecular Na^+ bridges between sulfate groups of the trisaccharide side-chains with ionic bonding and electrostatic forces of attraction on different molecules may take place resulting in gel-formation, because $\tan \delta$ values showed high values [48].

In Tris-HCl buffer solutions, the sulfate groups of rhamnan sulfate molecules associated with amino groups (NH_2) through ionic bonding and electrostatic forces of attraction between the sulfate groups of the trisaccharide side-chains on different molecules, resulting in gel-formation, because $\tan \delta$ values also increased in Tris-HCl buffer [48].

5. Conclusion

The α -1,3-linked rhamnan sulfate from *Monostroma nitidum* gelled in the presence of Tris (2-amino-2-hydroxymethyl-propane)-HCl, but α -1,4-linked ulvan from *Ulva pertusa* made a gel via Ca^{+2} cation [43,44]. Those different results are caused by molecular structure as shown in Figure 2 and 1.

Thus, rhamnan sulfate from *Monostroma nitidum* has unique gelling characteristics. The results and discussion suggests that rhamnan sulfate can be used in foods, cosmetics and some other industries as a gelling, thickening, stabilizing and water-holding agents. Finally, rheological analysis is the most useful method to discuss the structure-function relationship of polysaccharides at the molecular level in aqueous solutions.

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The Author has also heard lot about Emil Fischer, van't Hoff, Max Planck, Hermann Nernst and Svante Arrhenius

because those Nobel Laureates were professors of Hans von Euler in Berlin, Göttingen and Stockholm Universities, Germany and Sweden. Professor van't Hoff is well known as a man who created stereo chemistry by finding out the carbon atom involves tetrahedral conformation, and relationship between stereo isomers and their optical activity. Professor Emil Fischer is also well known as a man who established stereo chemistry by elucidating chemical structure of D-glucose, D-galactose, D-mannose and D-fructose. Professor Nernst and Arrhenius discovered dissociation of electrolyte in water and third law of thermodynamics.

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