

# Structure and Rheological Behaviour of Agar Extracted From Madagascar Sea Coast Algae

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**Abstract:** Polysaccharide from an original red algae (*Gelidium* sp) was extracted with hot water, clarified, and precipitated in presence of ethanol. NMR spectroscopic analyses and chromatographic separation after complete hydrolysis showed that this polysaccharide was composed predominantly of a repeating-unit based on 4-*O*-3,6-anhydro- $\alpha$ -L-galactopyranose and 3-*O*- $\beta$ -D-galactopyranose confirming their agar nature. The *Gelidium* sp agar (representing 16 w % on the basis of dried crude algae) has a regular chemical structure and forms strong gels in aqueous medium. Its rheological behavior is examined as a function of polymer concentration and temperature.

**Keywords:** Red algae, Agarose, Hydrolysis, Gelation, <sup>1</sup>H NMR, Rheology.

## 1. INTRODUCTION

Agar consists of a series of polysaccharides extracted from *Rhodophyta* seaweeds (red algae). They are currently used for many biological and industrial applications [1-3] having also an important role in various areas of emerging biotechnology [4]. Usually, the neutral fraction named agarose, being soluble in boiling water but insoluble in cold water, is extracted with water at high temperature. Agars are commercially important due to their gel-forming properties. It is generally agreed that the basic repeat unit of this polysaccharide is agarobiose which consists of 4-*O*-3,6-anhydro- $\alpha$ -L-galactopyranose (A unit) and 3-*O*- $\beta$ -D-galactopyranose (B unit) [5]. Variable amounts of substituent groups are present along the polysaccharide chains in the different fractions of agar, the most frequent substituents being methoxyl, sulphate esters and pyruvate ketal groups [6]. Sulphate groups are located at C-4 of D-galactose units (B unit) and C-6 of L-galactose units (A unit), methoxyl groups are located at C-6 of D-galactose (B unit) and C-2 of L-galactose residues (A unit) [7]. Moreover, L-galactose-6-sulphate residues are often present as a partial replacement of 3, 6-anhydro-L-galactose [8-12]. Many red algae contain more complex galactans devoid of gelling ability. In addition, several red algae may contain sulphated mannans or neutral xylans instead of sulphated galactans as the main structural polysaccharides [13].

The gel-forming properties of agar are directly related to the presence of the 3,6-anhydro-L-galactopyranose residues due to the three equatorial hydrogen atoms and cycle constrains allowing the molecule to form a double helix [14]. The association of the double helices is the basis of the thermoreversible gel formed with agarose and also  $\kappa$ - and  $\iota$ -carrageenans extracted from red algae [15]. The ability to form gels is widely influenced by the chemical substituents [16,17] as well as by the molar mass and molar mass distribution of the agar polymers [18]. The conformational analysis of agarose (pure AB copolymer) also predicts single and double helices [19, 20]. The purpose of the present work is to determine the chemical structure and rheological properties of agar extracted from an original species of red algae isolated from the Madagascar coast.

## 2. EXPERIMENTAL

### 2.1. Materials

The agar investigated was extracted from a red algae (*Gelidium* sp, named RHT) collected from Fenerive-Est, in east coasts of Madagascar; the corresponding strain is not yet completely identified. After collection, specimens were washed under tap water, cleaned by hands to get out of epiphytes and other impurities, dried in a Binder Drying oven (R3, type FD 53 Germany) at 50 °C for 1 day and ground in a ultraturrax Janke- Kunkel type MFC, IKA (Germany), stitch 1mm.

Myo-inositol with a purity better than 99.5 % was provided by Fluka, dimethylsulfoxide (DMSO) with a purity of 99.9 % and sodium borohydride >96 % (NaBH<sub>4</sub>) by Sigma-Aldrich. HCl 37 %, SO<sub>4</sub>H<sub>2</sub> 96 %, ethanol 96.2 ° and

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methanol are from Carlo Erba. D<sub>2</sub>O was purchased from Eurisotop (France).

## 2.2. METHODS

### 2.2.1. Extraction

3 grams of dried crushed seaweed were stirred in distilled water on a Fisherbrand magnetic stirrer hot plate, model L-81 and placed in water bath JP Selecta s.a.(Spain) at 100 °C under reflux for 2 hours. The hot mixture was filtered immediately on Whatman filter papers (1,2 3, diameter 110mm) at high temperature to prevent clogging; the filtrate was added, at room temperature, with an equal volume of distilled EtOH (90°) and let overnight at 4 °C in a refrigerator/freezer (Sharp, 431L) to allow complete precipitation. The precipitate obtained was washed with ethanol, and dried in a Binder Drying oven (R3, type FD 53, Germany) at 50 °C for 1 day. *Gelidium* sp extracted and purified polysaccharides represented 16 % on the basis of dried ground algae. The polymer extracted was named “agar” in the following because it may contain chains with different substituents.

### 2.2.2. Elemental Analysis

Elemental analysis used to determine the nitrogen, carbon and sulphur contents was realized by the ‘Service Central d’Analyse’ from CNRS (Solaize, France). Sulphur content was related with the presence of sulphate substituents and nitrogen to the presence of proteins.

### 2.2.3. Gas Chromatography

The polysaccharide was hydrolysed with 2 M H<sub>2</sub>SO<sub>4</sub> for 4 h at 100 °C in the presence of inositol as internal standard. The hydrolysate was neutralized with barium carbonate, deionized and the sugars were then reduced with an excess of NaBH<sub>4</sub> for 16 h at room temperature. Borate compounds were eliminated by MeOH/HCl 1% v/v treatment. The reduced sample was acetylated with acetic anhydride/pyridine (v/v) at 100 °C for 1h. After cooling and dilution with water, the mixture was evaporated to dryness. At end, the alditol acetate derivatives prepared were analyzed by gas chromatography equipped with a flame-ionisation detector (CPG/ FID) on a SP2380 macrobore column (0.53 mm x 30 m) in a Hewlett Packard 5890A system using various alditol acetate carbohydrates as standards. The carrier gas was high-purity nitrogen and the injector port was heated at 250 °C. For sample separation the following conditions were applied: 4 min at an initial temperature of 195 °C followed by an incremental increase (2.5 °C/min) to a final temperature of 225 °C during 2 min.

### 2.2.4. NMR Spectroscopy

<sup>1</sup>H NMR spectra of extracted polysaccharide was recorded at 85 °C on 10 mg/ml solution in D<sub>2</sub>O. These experiments were performed using a Bruker Avance 400 spectrometer operating at 400.13 MHz. <sup>1</sup>H chemical shifts were measured in parts per million, at 85 °C and the chemical shifts were referred to tetramethylsilane (TMS). Firstly, the methoxyl and pyruvic acid ketal contents were

quantified. Then, <sup>1</sup>H NMR spectrum of *Gelidium* sp agar was performed at different temperatures from 30 to 85 °C to evidence possible change in polysaccharide conformation.

### 2.2.5. Gel Characterization

Gels were casted from a heated aqueous solution of the total agar extracted from *Gelidium* sp (10 g/L) into cylindrical cuvetts. Cylindric pieces of gel with a 2.4 cm diameter and a thickness varying between 2 and 5 mm were recovered after cooling. Rheological properties of gels were obtained with a TA Instruments rheometer (AR2000) by dynamic measurements with parallel plates geometry giving the evolution of dynamic moduli (G', the storage modulus and G'', the loss modulus) as a function of the frequency in the linear domain. A Peltier plate allowed varying the temperature (temperature ramp 5 °/min) to determine the melting/gelling temperatures of agar gels. At low agar content, a cone-plate geometry was used with a 4 cm diameter and 3.59 ° angle cone.

## 3. RESULTS AND DISCUSSION

It seems particularly important to establish the details of the chemical structure of the crude extract from *Gelidium* sp forming easily strong gels in aqueous medium.

### 3.1. NMR Spectroscopy

NMR spectroscopy of the initial polymer confirmed that agarobiose was the main repeating unit in the polysaccharides extracted from *Gelidium* sp [13, 21] (Fig. 1). The signal at 1.52 ppm in the <sup>1</sup>H NMR spectra was assigned to the methyl group of pyruvic acid ketal linked to the 4- and 6- positions of β-D-galactopyranose residues [22]. Moreover, the small signal at 5.29 ppm was attributed to the anomeric proton of the L-galactopyranose residue, being deshielded by the adjacent pyruvate substituent. The signals appearing at 3.48 and 3.52 ppm were assigned to the methoxy groups of 6-*O*-methyl-D-galactose and 2-*O*-methyl-3,6-anhydro-L-galactose respectively [23].

At end, <sup>1</sup>H NMR of the *Gelidium* sp agar was studied as a function of temperature to evidence a possible conformational change (spectra not shown); it is known that, in the helical conformation, the signals are very broad and develop when temperature increases in relation with the conformational change to coil. The spectra obtained in the presence of a small amount of DMSO taken as reference clearly indicated, as reflected by the agar signals amplitude increases, that the mobility of the polysaccharide increased when temperature increased corresponding to progressive melting of the aggregates of double helices evidenced by optical rotation and DSC [24]. These results are discussed later in relation with the gel properties.

### 3.2. Chemical Composition of Crude Extracts.

Elemental analysis on the agar sample allows determining the carbon (C), nitrogen (N) and sulphur (S) percentages to test the purity of the samples and elements of global composition. Data are given in Table 1.

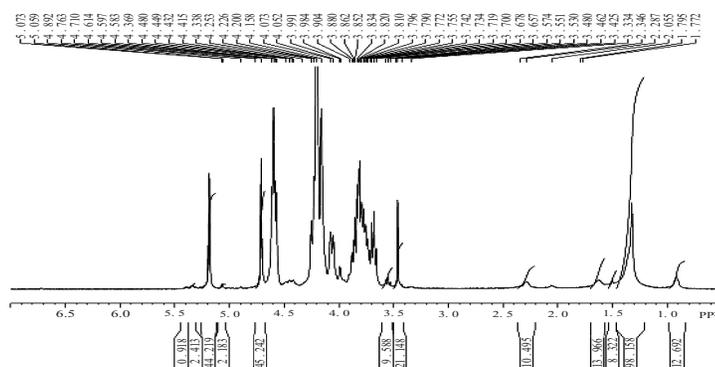


Fig. (1).  $^1\text{H}$  NMR of *Gelidium* sp (RHT) agar in  $\text{D}_2\text{O}$  at  $85\text{ }^\circ\text{C}$ .

Table 1. Elemental Analysis on the RHT Agar Sample

Element Determined in w%	<i>Gelidium</i> sp
C	37.4
N	<0.3
S	0.55

The estimation based on the dimeric repeat unit of pure agarose should give C % = 42.5 in relatively good agreement with the value found; the very low yield in nitrogen signified that there was negligible amount or no protein. The sulphur content allowed to evidence the low content of sulphate ( $-\text{SO}_3\text{H}$ ) substituents. Then, after analysis of hydrolysed polysaccharides with  $\text{H}_2\text{SO}_4$  by gas chromatography of the alditol acetates, the monosaccharide composition was obtained (Table 2).

Table 2. Composition of *Gelidium* sp Agar by Gas Chromatography

Sugar Units (w %)	<i>Gelidium</i> sp
galactose	82.3
glucose	2.5
6-O-Me-Gal	15.1
2-O-Me-Gal	~ 0

The presence of a low content of glucose in the crude extract seems to be related to some contamination.

### 3.3. Characterization of the Gel

The initial *Gelidium* sp agar formed gel in aqueous medium; it was investigated in the following. 0.1 M KCl solution was used as solvent to prepare gels for screening possible electrostatic repulsions which should be due to the presence of few sulphates in the total extract, as evidenced by sulphur titration (see Table 1).

#### 3.3.1. Influence of Polymer Concentration

The influence of polymer concentration (from 1 to 15 g/L) was studied to determine the critical concentration for gel formation. The experimental data are given in Table 3.

At  $25\text{ }^\circ\text{C}$ , the 1 g/L system remained a solution with macrogels ( $G'$  and  $G''$  were nearly superimposed indicating the limit for gelation and they were increasing with frequency), but at 3 g/L, a very soft gel was obtained; both samples were tested with a cone-plate geometry. The higher concentrations gave strong gels (tested with a plate-plate geometry) for which, the moduli increased rapidly with polymer concentration. The rheological behaviour in 0.1M KCl is given in Fig. (2).

At polymer concentration  $C \geq 3\text{ g/L}$ , a dependence of  $G'$  moduli with the frequency (of the order of  $G' \sim C^2$ ) was observed as previously found for  $\kappa$ -carrageenan and gellan [25]. Between 1 and 3 g/L, a change in the behaviour was evidenced: at 1 g/L,  $G' < G''$  at  $25\text{ }^\circ\text{C}$  but at 3 g/L,  $G' > G''$ . For this specific agar extract, we deduced a critical concentration for gelation located around  $c \sim 2\text{ g/L}$  in 0.1 M KCl at  $25\text{ }^\circ\text{C}$ .

#### 3.3.2. Influence of the Temperature

The temperature increase was imposed up to  $85\text{ }^\circ\text{C}$ . The evolution of  $G'$  moduli are given for different polymer concentrations in Fig. (3) allowing to determine the melting temperature for the gels. For 1 g/L, the value were extremely low with no significant dependence on temperature and no uniform gelation. At 3 g/L, the gel moduli decreased when temperature increased over  $45\text{ }^\circ\text{C}$ . Larger effect was observed at 10 g/L:  $G'$  was much larger than  $G''$  (as shown in Fig. 2) at low temperature and decreases strongly over  $50\text{ }^\circ\text{C}$ ; a crossover of  $G'$  and  $G''$  was obtained at  $83\text{ }^\circ\text{C}$  corresponding to the melting of the gel (data not shown for clarity of the figure). On the same sample, on temperature decrease,  $G'$  started to increase around  $50\text{ }^\circ\text{C}$  (the  $G', G''$  crossover was located at  $55\text{ }^\circ\text{C}$ ) corresponding to the progressive gelling of this polymer. The large hysteresis observed for a temperature cycle characterized this agar sample indicating a large degree of regularity in the chemical structure. From these data, it was also shown that the melting temperature increased when polymer concentration

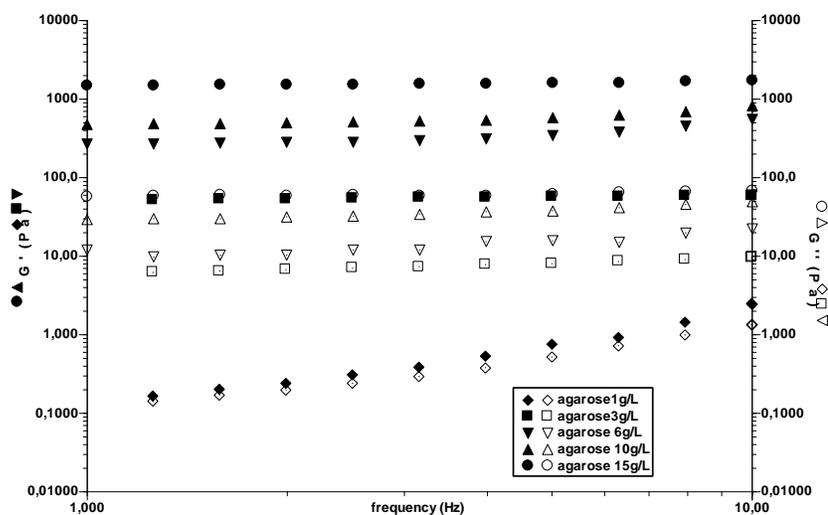


Fig. (2). Influence of polymer concentration on the gel moduli ( $G'$  filled symbols;  $G''$  open symbols) at 25 °C in 0.1M KCl.

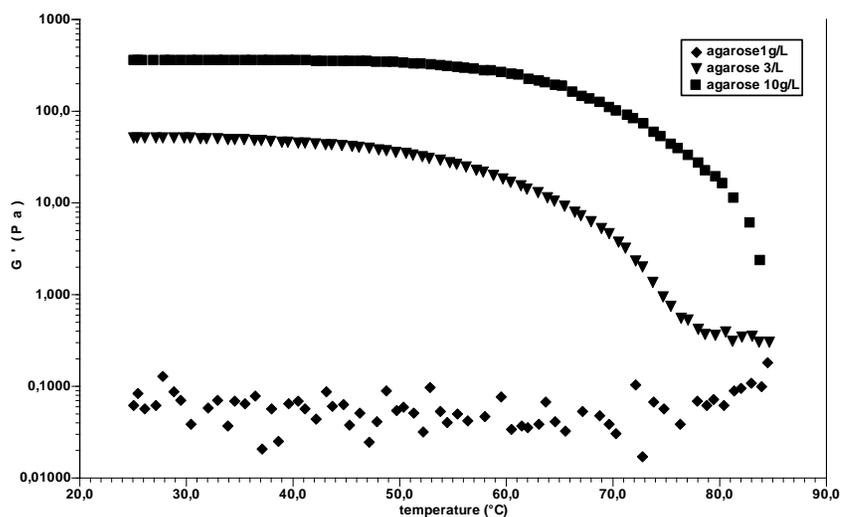


Fig. (3). Influence of temperature increase on the  $G'$  modulus measured at 1 Hz for agar at different concentrations.

Table 3. Rheological Characteristics of *Gelidium* sp Agar Gels Formed in Aqueous Solutions at Different Polymer Concentrations and Tested at Different Temperatures

C (g/L) /Temperature	$G'$ (Pa) at 1Hz	$G''$ (Pa) at 1Hz	$G'/G''$ (at 1 Hz)
1 at 25 °C	0.146	0.124	1.2
3 at 25 °C	50.52	6.03	8.4
3 at 85 °C	0.30	0.23	----
6 at 25 °C	446.5	21.5	20.8
10 at 25 °C	639.5	23.7	27
10 at 85 °C	0.70	1.9	----
15 at 25 °C	1464	55.9	26.2

increased: located around 77 °C at 3 g/L, it shifted to 83 °C at 10 g/L where  $G'=G''$ . The high temperature of melting was in agreement with the chemical composition of *Gelidium* sp agar containing a low yield in substituent.

Considering the evolution of the NMR spectra with temperature, it was clear that the decrease in  $G'$ (Pa) down to melting was related to the increase of the H-1 signal for galactose unit relative to DMSO (at 2.8 ppm) used as reference. Temperature increased progressively the mobility

**Table 4. Influence of the Temperature on the Storage Modulus (G') and NMR Mobility**

Temperature (°C)	G' (Pa)	Gal/DMSO Relative NMR Signal Area
25	356.7	0.74
45	345.4	1.34
65	191.4	1.56
85	0.7	2.83

The gel melting is known to be related to the helix-coil transition as confirmed by the increases in chain mobility.

of the polymer initially packed as double helices aggregates (Table 4)

#### 4. CONCLUSION

Red algae (Rhodophyta) are known as source of unique sulfated galactans, such as agar, also named agarose in absence of sulphated units. The wide practical uses of these polysaccharides are based on their ability to form strong gels in water or in KCl solutions.

In a first part of this work, the chemical structure was briefly described using <sup>1</sup>H NMR and gas chromatography after total hydrolysis. The extract from original algae named *Gelidium* sp (16 w % on basis of the dried algae was recovered) was built up of the characteristic repeating disaccharide units found in agarose with a low yield in methyl substituents on the A unit.

In a second step, the rheological properties of the extract were studied: this *Gelidium* sp extract formed strong gels in water and in presence of 0.1 M KCl. For this original agar the critical concentration for gelation was determined around 2 g/L. The influence of temperature characterized the thermoreversible gel with a large hysteresis at 10 g/L and a melting temperature at 83 °C in the experimental conditions adopted.

These results demonstrated that this new algae collected along Madagascar coasts has a great potential for a future development.

#### CONFLICTS OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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