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# Structural and compositional characteristics of gelling galactan from the red alga *Ahnfeltia tobuchiensis* (Ahnfeltiales, the Sea of Japan)

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**Abstract**

The composition and structure of a sulfated polysaccharide from *Ahnfeltia tobuchiensis*, a red seaweed of Kuril Islands and Japan, were investigated. Samples of the galactan were characterized by $^{13}C$ NMR and FTIR spectroscopy and chemical analysis in comparison with well-known commercial agarose preparations. The main components of the polysaccharide from *A. tobuchiensis* were 3,6-anhydro-L-galactose (38 ± 4.5%) and galactose. Methoxy groups (2.2%) of the galactan are located in the position 2 of the anhydrogalactose residues. The galactan proved to be a high-sulfated agarose. Sulfur content of this polysaccharide is 0.2–0.3%, and sulfate groups may be removed by alkali treatment to a limited extent only. The precision of quantification and possibilities of analytical methods used are discussed.


*Keywords:* Agarose; *Ahnfeltia tobuchiensis*; Methylation degree; Sulfated polysaccharide, NMR spectroscopy

## 1. Introduction

Red algal galactans are sulfated polysaccharides having usually a linear backbone built up of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues. The β-galactose residues always belong to D-series, whereas the α-galactose residues are D in carrageenans and L in agars. A substantial part of the α-galactose residues may exist in the form of a 3,6-anhydro derivative. Using the nomenclature of Knutsen, Myslabodski, Larsen, and Usov, (1994), the galactose and anhydrogalactose residues in agars are referred to as G and LA units in agars (G and DA in carrageenans), respectively (Fig. 1). Various hydroxy groups may be substituted by ester sulfate, methyl groups, pyruvic acid acetal and sometimes by additional monosaccharide residues (Painter, 1983; Usov, 1998).

From more than 4000 red algae (Rhodophyta) species known at present, only about 70–80 species are used for industrial production of gelling galactans. The most important ones belong to the 2nd subclass (Florideae) of Rhodophyta division, orders Gelidiales and Gigartinales and mainly genera *Ahnfeltia*, *Chondrus*, *Eucheuma*, *Furcellaria*, *Gelidiella*, *Gelidium*, *Gigartina*, *Gracilaria* and *Pterocladia*. From the genus *Ahnfeltia*, six main species are known, in both cold and warm regions. Depending on climatic conditions, some species of *Ahnfeltia* can produce agar as well as carrageenan type galactans (Levring, Hoppe & Schmid, 1969).

One of the economically most important Ahnfeltia species is *Ahnfeltia plicata* (Huds.) Fries., frequently mentioned as a raw material for Russian or Sakhalin agar in Russia and for Itani agar in Japan. *A. plicata* and/or varieties of the species supply in total (in Russia) some 170 thousand tons of seaweeds mainly from Peter the Great Bay (near Vladivostok) and Izmen Bay (south of Kunashir island, the Kurils) (Chapman & Chapman, 1980; Levring, Hoppe, & Schmid, 1969). This is the only alga along the whole Far-East coastal region from Chukotsk Peninsula to Vladivostok, being used for industrial production of gelling algal polysaccharides. During the last two decades of the 20th century it was established that three species of the genus *Ahnfeltia* are distributed in the Far East seas of Russia, and the resource of algae has become rather more abundant (Titlyanov, Cherbadgy, & Chapman, 1999). *Ahnfeltia* species are technologically the most valuable seaweeds on the whole territory of Russia (Vozzhinskaja, Tzapko, Blinova, Kalugina, & Petrov, 1971). *A. plicata* is a typical seaweed species capable

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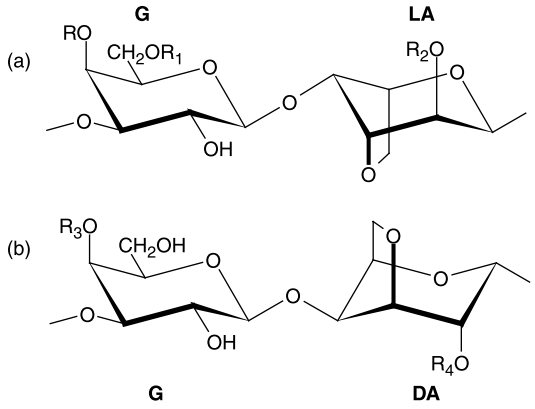


Fig. 1. Basic disaccharide repeating units of (a) agarose; and (b) some carrageenans. (a) R = H or $SO_3^-$, $R_1$ = H or Me or $SO_3^-$, $R_2$ = H or Me; (b) $R_3$ = $SO_3^-$, $R_4$ = H or $SO_3^-$ (kappa- and iota-carrageenans).

of producing galactans of agar or carrageenan type depending on environmental conditions. For example, this alga contains carrageenans in North American and Irish waters but agar-type polysaccharides in the White Sea (Levring et al., 1969; Kollist, 1981).

The name *Ahnfeltia tobuchiensis* for one of Ahnfeltiales of Russian Far East was introduced by Makijenko (1970), precedingly this seaweed was defined as a variety: *A. plicata* var. *tobuchiensis* (Kanno & Matsubara, 1932). At present, the name *A. tobuchiensis* (Kanno et Matsubara) Makijenko is officially recognized (Taxonomical Index 1996).

As mentioned above, this Far East alga species (under names *A. tobuchiensis* or *A. plicata*) is being used for production of gelling polysaccharides. During the last years, distribution of biomass and primary production of this species (Cherbadgy & Popova, 1998; Titlyanov et al., 1999) as well as seasonal influences on chemical composition of the seaweeds (Kostetsky, Goncharova, Sanina & Shnyrov, 2004) are widely investigated. Also some rheological and compositional aspects of the galactan mixture from this seaweed have been studied (Kollist, Vaher, Truus, Paris, & Püssa, 1989). Inorganic composition (mineral part) of this natural polysaccharide has been detally analysed (Truus, Taure, Eglite, Limberg, Ivask and Vaher, 1993). Investigations of Kollist (1981) by comparative fractional extraction of the seaweeds confirmed the expediency to differentiate *A. tobuchiensis* as an individual species. Later also some galactan fractions for laboratory applications from this seaweed are separated (Sukhoverkhov, Kadnikova, & Podkorytova, 2000).

Polysaccharide mixtures isolated from this species have a more or less homogeneous composition depending on preceding fractionation procedures. Commercial products on the basis of *A. tobuchiensis* have been manufactured mainly as a gelling material for food industry (in Russian Far East and in Japan) or in the form of more narrow fractions for separating media used in gel filtration and affinity chromatography (in Estonia). Although these various galactan fractions have often been referred to as agar and agarose and utilized as gelling agents, the exact chemical nature and structural specifity of this natural matrix have never been established.

In this work, some aspects of composition of the galactan matrix from *A. tobuchiensis*, such as sulfur content and variability and location of methoxy groups, are investigated. The main purpose of this research is to elucidate the principal structural feature of the matrix from the red alga *A. tobuchiensis* in comparison with well-known commercial preparations of agarose.

## 2. Experimental

### 2.1. Materials

Galactan mixtures from *A. tobuchiensis* (various lots of commercial production) were obtained from Pilot-production Plant of the Institute of Chemistry, Estonian Academy of Sciences. In technological process, the galactans were isolated from seaweeds *A. tobuchiensis* originated from Russian Far East (Peter the Great Bay near Vladivostok and Izmen Bay of Kunashir Island) by fractional extraction in a weak-alkaline solution. Calcium hydroxide suspension (25 g CaO per 1 kg of algae) was used as alkali, and the most electroneutral fraction of algal galactans was separated to produce gelling agents. A product called agar was isolated by freezing-thawing procedure and additionally fractionated by polyethylene glycol (MW 6000). The manufacturing process corresponded to a certain patent specification (Kollist & Püssa, 1981).

Agarose preparations for comparative study were purchased from LKB (medium EEO) and Sigma (type I-A, low EEO).

### 2.2. Chemical analysis

Sulfur content was determined by a turbidimetrical method (Dodgson, 1961), slightly modified by us. Turbidity was characterized by measurements of optical density (1 cm pathlength) on a Shimadzu UV-1601 spectrophotometer.

Methoxy group content of galactans was analyzed by a classical method (Cheronis & Ma, 1964) and a standard microchemical apparatus (Steyermark, 1956). Nevertheless, some modifications have been made to compare the action of liquid and solid sorbents used in the apparatus.

3,6-Anhydrogalactose content was established according to Yaphe and Arsenault (1965) using fructose as standard sugar.

The total carbohydrate content was estimated by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) without previous hydrolysis of the polysaccharide.

### 2.3. Alkaline treatment

Treatment of galactan mixture from *A. tobuchiensis* with alkali was performed according to Lönnerdal and Låås (1976) using $NaBH_4$ to create a reducing medium.

### 2.4. Electroendoosmosis (EEO)

EEO characteristics ($-m_r$) of 1% galactan gels in 0.05 M Tris–barbital buffer (pH 8.6) were measured using a 2117

Multiphor electrophoresis block and 2197 Power Supply (LKB). Human serum albumin stained with bromophenol blue and vitamin $B_{12}$ served as anionic and neutral markers, respectively (Wieme, 1965).

### 2.5. IR spectroscopy

Infrared spectra were scanned with a PerkinElmer FTIR System Spectrum BX spectrometer from thin (0.015 mm) galactan films obtained by slow evaporation of 0.7–1% water solutions in polystyrene petri dishes at room temperature; 12 scans were recorded with resolution 4 $cm^{-1}$ and interval 1 $cm^{-1}$.

### 2.6. NMR spectroscopy

Proton-decoupled $^{13}C$ NMR spectra were recorded on a Bruker AMX-500 spectrometer for 4% galactan solutions in $H_2O$ mainly at 50 °C at a carbon frequency of 125.7 MHz, using delay 2.0 s and a 33° pulse angle. A small amount of $D_2O$ was added for the lock signal and mostly about 1000–4000 transients were collected before the Fourier transform. Chemical shifts are quoted relative to internal $Me_2SO$ at 39.47 ppm.

## 3. Results and discussion

### 3.1. Sulfur in the matrix

The usual sulfur content of the commercial galactan from *A. tobuchiensis* (ATG) is 0.2–0.3%. Parallel investigations of different lots showed that this value slightly varies between these values depending on raw material and differences in processing conditions, particularly on duration of washing of the final product. Such a sulfur content is quite high for agarose (if there is an agarose fraction in ATG) for which the content 0.6% $SO_4$ (or 0.2% S) is the highest conventional limit (Duckworth & Yaphe, 1971). Prolonged water elution of the galactan preparations reduces sulfate content (high-charged fractions get faster detached), but this process is disadvantageous for practical applications.

Sulfur content of ATG may be decreased by alkali treatment to a limited extent only. IR spectra (Fig. 2) show an insignificant change after this treatment in the region of 820–850 $cm^{-1}$, corresponding to oscillations of COS-groups in the galactopyranose cycle (Anderson, Dolan, Penman, Rees, Mueller and Stancioff, 1968). Bands at other wavenumber values characteristic to sulfur-containing groups in red-algal galactans at 805–810 $cm^{-1}$ as representative of axial 2-sulfate groups and 1250 $cm^{-1}$ for total sulfate (Lloyd & Dodgson, 1961; Whyte, Hosford, & Englar, 1985) do not show any substantial change after alkali treatment. Also the EEO characteristics have only a low decreasing tendency in alkaline medium. The $-m_r$ values turned out to be as follows: for the initial galactan 0.22, after alkali treatment 0.21, after additional alkali treatment(s) 0.19 as a minimum. This little removable part of sulfur present in the matrix should be located in the G-6

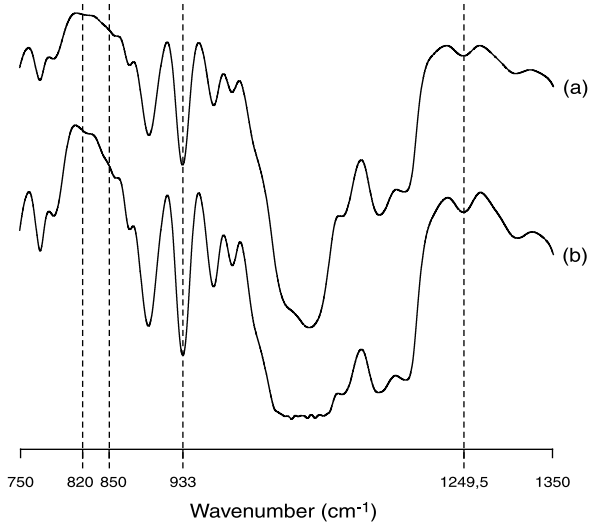


Fig. 2. FTIR spectra of the commercial galactan from *A. tobuchiensis* (a) before; and (b) after alkali treatment.

position (Turvey, 1965). The above-given calculations on sulfur content are in accordance with more recent investigations (Matsuhiro, 1996; Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003). Thus, it may be concluded from the data that the sulfate groups causing polyelectrolyte properties of the ATG chain are located in different positions of disaccharide residues and cannot be removed to a great extent from the galactan matrix. That is, the preceding industrial process had been nearly sufficient to separate and convert the charged fraction of ATG.

Still there were some sorbents, especially γ-$Al(OH)_3$ gel, used (not shown) for selective binding of high-charged galactan fractions to reduce the $-m_r$ values of ATG. However, the results vary depending on differences of natural raw material. Probably, some more specific desulfation method such as the use of chlorotrimethylsilane (Kolender & Matulewicz, 2004) will appear more suitable.

### 3.2. Monosaccharide content and the composition of ATG

The mean value of 3,6-anhydrogalactose content of ATG is 38% and may significantly (±4.5%) vary with the lot of the product. The anhydrogalactose share may be determined also from a strong band at 930–940 $cm^{-1}$ (933 $cm^{-1}$ in our case, see Fig. 2) in FTIR spectrum (Stancioff & Stanley, 1969). The total carbohydrate content of ATG was 96±3%. The results are in accordance with earlier data about the content of galactose (58%) (Kollist, 1981) and ash (0.27%) (Truus et al., 1993) in ATG. It is evident from the NMR spectra (Fig. 3) that there is no saccharide residue in addition to those of galactose and anhydrogalactose in ATG.

### 3.3. Methoxy group analysis

Methylation degree of hydroxyl groups causes the most significant differences in structure of various agarose matrices. The values of methoxyl content of LKB and Sigma agaroses

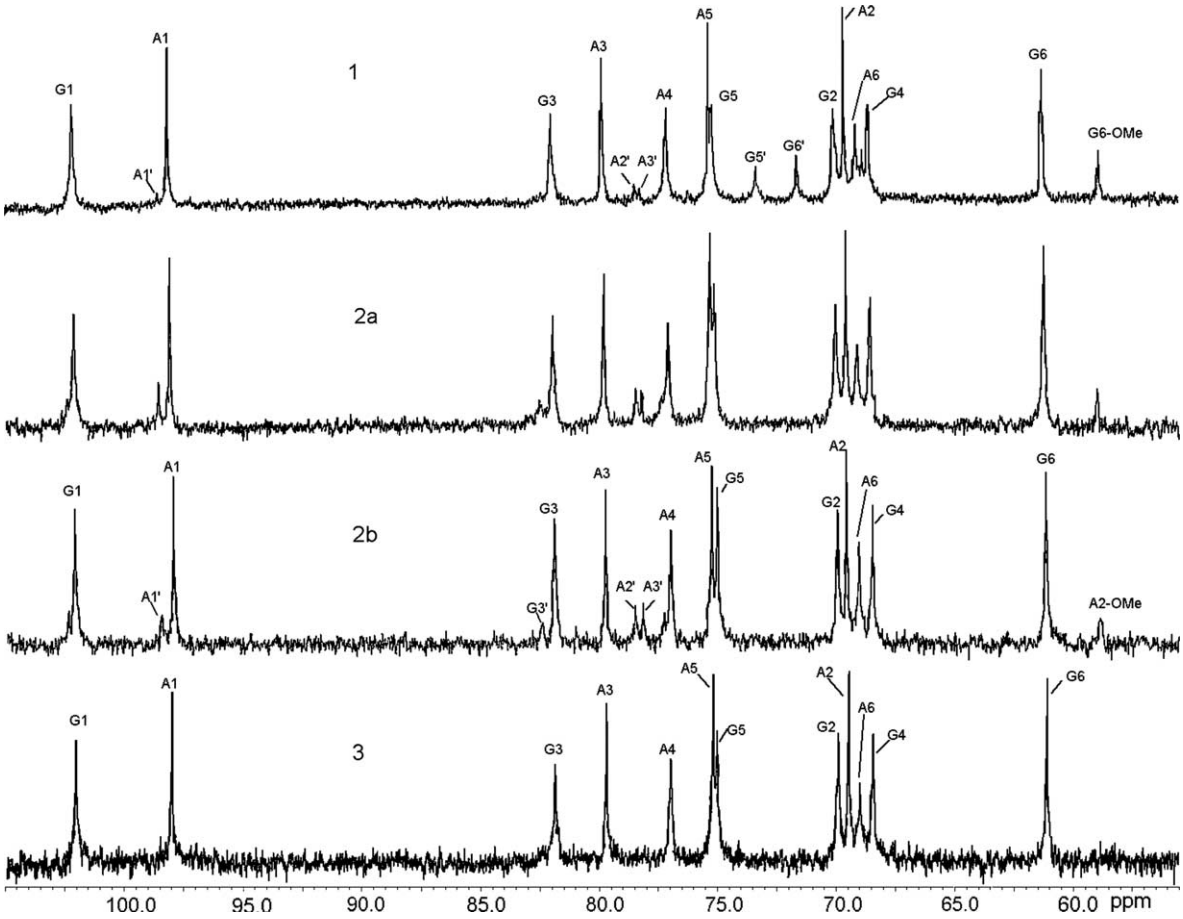


Fig. 3. $^{13}C$ NMR spectra of some commercial galactan preparations (recorded at 50 °C, 2b at 70 °C): (1) Agarose LKB, 1600 transients; (2) galactan from *A. tobuchiensis*, 1000 transients (2a) and 700 transients (2b); (3) Agarose Sigma, 3900 transients collected.

used for further structural investigations in comparison with ATG are presented in Table 2. The real accuracy of determinations by microchemical methoxy group analysis (measured in triplicate) of galactans has two significant figures (if $CH_3O$ content $>1\%$) or one significant figure (if $CH_3O$ content $<1\%$). It was established that this approximate rule is valid in the case of liquid as well as of solid sorbents used in the course of standard microchemical analysis. Deviation of the parallel investigations is caused mostly by uneven reaction process, particularly at low methoxyl content.

### 3.4. C-NMR spectroscopy

$^{13}C$-NMR spectra of three galactans investigated are presented in Fig. 3. The respective chemical shifts of basic carbon atoms in the three samples, allowing to establish firmly

Table 1
$^{13}C$-NMR chemical shifts (ppm) for basic signals of carrageenan and agar structures

| Unit | $^{13}$Carbon chemical shift | | | | | | Ref. |
|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | |
| G | 102.5 | 69.5 | 80.4 | 66.4 | 75.3 | 61.3 | Usov, & Shaskov, 1985 |
| DA | 94.7 | 70.2 | 79.4 | 78.0 | 76.8 | 69.5 | |
| G | 102.4 | 70.2 | 82.2 | 68.8 | 75.3 | 61.4 | Lahaye, Yaphe, Viet, & Rochas, 1989 |
| LA | 98.3 | 69.9 | 80.1 | 77.4 | 75.7 | 69.4 | |
| G | 102.3 | 70.1 | 82.2 | 68.6 | 75.2 | 61.3 | Usov, Yarotsky, & Shashkov, 1980 |
| LA | 98.2 | 69.7 | 80.0 | 77.2 | 75.5 | 69.7 | |
| G | 102.07 | 69.98 | 81.92 | 68.52 | 75.09 | 61.20 | Observed galactan (ATG) |
| LA | 98.05 | 69.55 | 79.77 | 77.04 | 75.25 | 69.05 | |
| G | 102.07 | 69.99 | 81.93 | 68.53 | 75.10 | 61.20 | Observed agarose (from Sigma) |
| LA | 98.05 | 69.56 | 79.77 | 77.04 | 75.25 | 69.07 | |
| G | 102.08 | 69.98 | 81.93 | 68.52 | 75.10 | 61.20 | Observed agarose (from LKB) |
| LA | 98.06 | 69.55 | 79.77 | 77.04 | 75.25 | 69.06 | |

Table 2
Methoxy group content and location in commercial galactan preparations

| Preparation | Chemical shifts, ppm[a] | Methoxy groups | | Content of methylated disaccharide units[b], % |
|---|---|---|---|---|
| | | Location | Summary content[b], % | |
| Agarose LKB, Medium EEO | 58.80 OMe | | | |
| | 71.53 G6′ | G6 | | |
| | 73.27 G5′ | | | |
| | | | 3.9 | 79 |
| | 78.16 A3′ | | | |
| | 78.38 A2′ | A2 | | |
| Galactan from *A. tobuchiensis* | 58.93 OMe | | | |
| | 78.17 A3′ | A2 | 2.2 | 45 |
| | 78.41 A2′ | | | |
| | 82.48 G3′ | | | |
| Agarose Sigma, Type I-A | No minor signal detected | Undeter mined | 0.9 | 18 |

[a] Minor signals (Fig. 3) caused by influence of methoxy groups.
[b] From mass of dry matter.

the structure type of galactans (Usov, Yarotsky, & Shashkov, 1980; van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002), are very close to each other (Table 1). The basic signals of 12 carbon atoms from disaccharide repeating unit of ATG are characteristic for agar-type polysaccharides and the galactan backbone from *A. tobuchiensis* is analogous to those of LKB and Sigma agaroses.

At the same time it can be observed (Table 1) that while in the case of parallel investigations the chemical shifts of agaroses coincide quite precisely, the results of various authors, concerning agaroses as well as other galactans, may differ considerably (see also Melo, Feitosa, Freitas, & de Paula, 2002).

The main structural and quantitative characteristics of commercial agaroses concern the location and content of methoxy groups. $CH_3O$ groups of ATG are located in 3,6-anhydro-L-galactose (LA) residues (Fig. 3, Table 2); D-galactose residues (G) are free from methylation. Methoxy groups of LKB agarose are divided between two positions of G and LA residues (Fig. 3, Table 2). The low methoxyl content of Sigma agarose (type I-A) does not allow to determine the location of the groups. Probably the value about 0.9–1% $CH_3O$ in algal galactans is the minimum detection limit for 500 MHz spectrometers at a reasonable recording time. Comparison of spectra 2a and 2b demonstrates how the temperature rise permits to reduce the number of scans.

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