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Short communication

Structural characterization of polysaccharide obtained from red seaweed *Gracilaria caudata* (J Agardh)

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ABSTRACT

Seaweeds are considered an important source of bioactive molecules. In this work the marine red alga *Gracilaria caudata* was submitted to aqueous extraction of their polysaccharides for 2 h at 100 °C. The polysaccharide fraction (PGC) presented a recovery of 32.8%. The sulfate content of PGC, calculated by *S*%, is 1 ± 0.2% and the degree of sulfation accounts for 0.13 ± 0.2. High-Performance Size-Exclusion Chromatography demonstrated that PGC consists of a high molecular weight polysaccharide (2.5×10^5 g mol^{-1}). Chemical analysis of PGC was performed by microanalysis, infrared (FT-IR) and nuclear magnetic resonance (NMR, 1 and 2D) spectroscopy. The structure of PGC is mainly constituted by the alternating residues 3-linked-β-D-galactopyranose and 4-linked-3,6-α-L-anhydrogalactose; however some hydroxyl groups were substituted by methyl groups and pyruvic acid acetal. The biological precursor of 3,6-α-L-anhydrogalactose (6-sulfate-α-L-galactose) was also detected.

1. Introduction

Marine macroalgae are an important source of sulfated polysaccharides, molecules widely used in the food industry because of their rheological properties as gelling and thickening agents (de Almeida et al., 2011; Mohamed, Hashim, & Rahman, 2012; Wijesekara, Pangestuti, & Kim, 2011). These compounds found in Rhodophyta are galactans consisting of galactose or modified galactose units which are classified as agarans and carrageenans based on their stereochemistry (Costa et al., 2010; de Almeida et al., 2011; Ferreira et al., 2012; Jiao, Yu, Zhang, & Ewart, 2011; Wijesekara et al., 2011).

The agarans and carrageenans exhibit a broad range of biological activities including antioxidant, gastroprotective, anti-inflammatory, antinociceptive, anticoagulant, anticancer, immunomodulatory, antiproliferative and antithrombogenic (Barahona, Encinas, Mansilla, Matsuhiro, & Zuniga, 2012; Costa et al., 2010; Coura et al., 2012; de Araujo et al., 2011; Jiang, Hama, Yamaguchi, & Oda, 2012; Jie, Zhang, Chen, Mao, & Tang, 2012; Lins et al., 2009; Silva et al., 2011; Souza et al., 2012).

The genus *Gracilaria* is widely spread into tropical and temperate regions and is one of the largest genera in the Gracilariaceae family (Marinho-Soriano & Bourret, 2005). Despite the first extractions and manufacturing of agar type polysaccharides had been employed in the *Gelidium* genus (Armisen, 1995), the genus *Gracilaria* contains some of the most important agar producer species (Marinho-Soriano & Bourret, 2005; Plastino, Ursi, & Fujii, 2004). The increasing commercial and scientific interest in agar from *Gracilaria* demands a chemical elucidation of these polymers and improved knowledge of the relationship between molecular structure and functional properties (Coura et al., 2012; Murano, 1995; Silva et al., 2011; Souza et al., 2012).

The molecular structure of agar polysaccharides is complex (Fig. 1). Polysaccharides from *Gracilaria* genus are composed mainly of alternating residues of 3-linked-β-D-galactopyranose (G unit) and 4-linked-3,6-anhydro-α-L-galactopyranose (LA unit) (Araki, 1966). Various hydroxyl groups may be substituted by ester sulfate, methyl groups and pyruvic acid with their position varying from species to species. The possible structural changes affect the physical and rheological properties of these polymers (Andriamanantoanina, Chambat, & Rinaudo, 2007; Freile-Pelegrin & Murano, 2005; Lahaye, 2001; Lahaye & Rochas, 1991; Lahaye & Yaphe, 1988; Melo, Feitosa, Freitas, & de Paula, 2002; Usov, 1998; Valiente, Fernandez, Perez, Marquina, & Velez, 1992).

The agarocolloid structures can be studied by different ways, including chemical methods as colorimetric reactions and physical techniques such as Fourier transformed infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy (Ferreira et al., 2012; Freile-Pelegrin & Murano, 2005; Lahaye, 2001; Maciel et al., 2008; Montano, Villanueva, & Romero, 1999; Souza et al., 2012).

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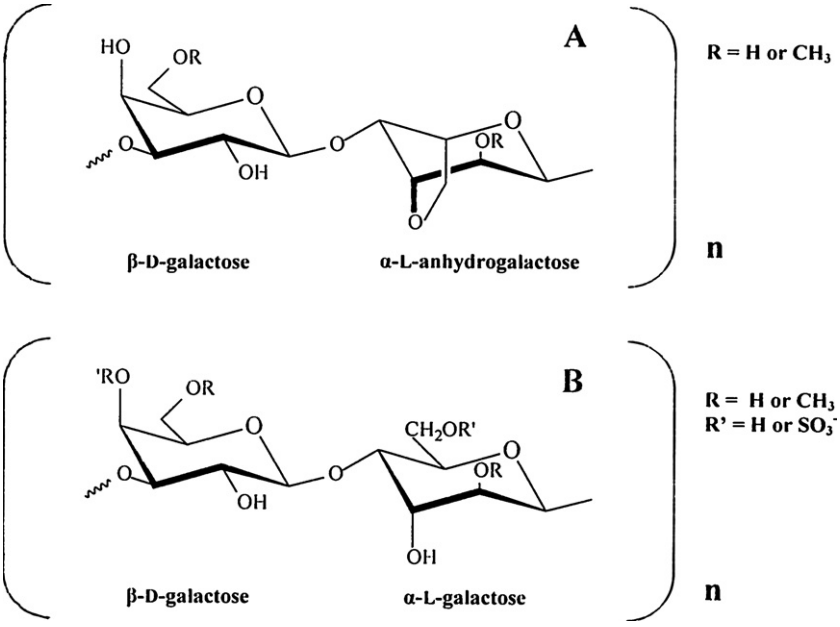

Fig. 1. Chemical structure of the repeating disaccharide unit of agar type molecules with the different types of sugar units and substituents.

Although different classification systems of agar-type galactans have been proposed, here we use the nomenclature and classification proposed by Knutsen, Myslabodski, Larsen, and Usov (1994).

In this work, chemical analysis, FT-IR, 1 and 2D NMR spectroscopy of ^{13}C and ^{1}H were used to determine the chemical structure of the polysaccharide fraction obtained from the red seaweed *Gracilaria caudata* J Agardh.

2. Experimental

2.1. Isolation of the soluble polysaccharide

Specimens of the red seaweed *G. caudata* were collected in August 2008 from the Atlantic coast at Northeast of Brazil (Pacheco Beach, Caucaia – Ceará). A Voucher sample of seaweed *G. caudata* specimen was deposited on Herbarium Ficológico do Instituto de Ciências do Mar at Federal University of Ceará-Brazil (no. 2384). The dried tissue of *G. caudata* (5 g) was ground into a fine powder and extracted in distilled water (1.5%, w/v) under stirring for 2 h at 100 °C. After filtration and concentration of the solution the polysaccharide was precipitated with ethanol (1:3, v/v) followed by washing with acetone and dried with hot air (Maciel et al., 2008). The polysaccharide fraction was then re-dissolved in distilled water (1.5%, w/v) and submitted to same protocol of precipitation, washing and drying. The polysaccharide was designated as PGC.

2.2. Composition

Nitrogen, carbon and sulfate content were determined by elemental microanalysis (Perkin Elmer CHN 2400). Protein content was calculated from N% using the correction factor of 6.25, as proposed by Marks, Buchsbaum, and Swain (1985). Moisture was obtained by heating 0.5 g of samples at 105 °C for 24 h (Maciel et al., 2008).

2.3. Molar mass distribution

The peak molar mass (M_{pk}) was estimated by High-Performance Size-Exclusion Chromatography (HPSEC) in a Shimadzu equipment at room temperature using an ultrahydrogel linear column (7.8 × 300 mm), flow rate of 0.5 ml/min, 0.5% polysaccharide concentration and 0.1 M $NaNO_3$ as solvent. A differential refractometer was used as detector and the elution volume corrected to ethylene glycol at 11.25 ml as an internal marker. A calibration curve was obtained by use of Pullulan (Shodex Denko) of different molecular weights ranging from 10^3 to 10^6 g mol^{-1} in order to estimate the peak molar mass (M_{pk}) for *G. caudata* polysaccharide. The equation obtained from this calibration plot was:

$$\log Mw = 14.64656 - 1.03196\,V_e \quad (1)$$

where V_e is the elution volume in ml. The linear correlation coefficient was 0.990.

2.4. Infrared spectroscopy

The Fourier transform IR spectra (FT-IR) were recorded with a Shimadzu IR spectrophotometer (model 8300) between 400 and 4000 cm^{-1}. The samples were analyzed as KBr pellets.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C and ^{1}H NMR spectra of 2.5% (w/v) solutions in D_2O were recorded at 353 K on a Fourier transform Bruker Avance DRX 500 spectrometer with an inverse multinuclear gradient probe-head equipped with z-shielded gradient coils, and with Silicon Graphics. Sodium 2,2-dimethylsilapentane-5-sulphonate (DSS) was used as the internal standard (0.00 ppm for ^{1}H). A distortionless enhancement by polarization transfer (DEPT 135) spectrum was recorded in order to determine the hydrogenation of each carbon; the acquisition and delay times were 1.0 s. 2D ^{1}H and ^{13}C HSQC spectra were carried out using the pulse programs supplied with the apparatus.

3. Results and discussion

The gelling polysaccharide of *G. caudata*, obtained from aqueous extraction, under heating, accounts for 32.8 ± 0.9% of the seaweed dry weight. The agar yield of the seaweed *G. vermiculophylla* (Vergara-Rodarte et al., 2010) and *G. dura* (Marinho-Soriano, 2001) were 28.0 and 33.5%, respectively. The yield of *G. caudata* was higher than that obtained for *G. birdiae* (6.5%; Maciel et al., 2008), *G. debilis*

(14.8%; Mehta, Meena, Prasad, Ganesan, & Siddhanta, 2010) and *G. cornea* (21.4%; Melo et al., 2002), but was smaller than the recovery obtained for the extraction of *Gracilaria chilensis* (40.0%; Tello-Ireland, Lemus-Mondaca, Vega-Galvez, Lopez, & Di Scala, 2011), *G. gracilis* (36.8–46.6%; Skriptsova & Nabivailo, 2009) and *G. cervicornis* (39.3%; Freire-Peregrin & Murano, 2005).

The nitrogen content of PGC was $1.4 \pm 0.3\%$ corresponding to $8.75 \pm 0.3\%$ of protein in the sample. The nitrogen result was similar to the 1.22% content stated for *G. birdiae* polysaccharide (Maciel et al., 2008) but lower than that obtained for *G. bursa-pastoris* (3.22%; Marinho-Soriano & Bourret, 2003). The *S*% and *C*% found were $1.00 \pm 0.05\%$, and $35.9 \pm 0.6\%$, respectively. The degree of substitution for sulfate (DS sulfate) was found to be 0.13 ± 0.01, calculated based on Melo et al. (2002), according to Eq. (2). The proposed equation is based on the agarobiose structure (Fig. 1), considering that DS sulfate is defined by the number of OSO_3^- or sulfur atoms per disaccharide repeating unit, which possess 12 carbon atoms.

$$\mathrm{DS} = \frac{\%S/\text{atomic mass of } S}{\%C/\text{atomic mass of } C \times 12} = 4.5\left(\frac{S\%}{C\%}\right) \quad (2)$$

The amount of sulfate presented by PGC was lower than the observed for other polysaccharides from *Gracilaria* species. The agar from *G. sjoestedtii* showed 2.3% of sulfate (Craigie, Wen, & VanderMeer, 1984) while the sulfate content presented in *G. domingensis* and *G. mammillaris* was 7.6 and 8.9%, respectively (Valiente et al., 1992).

The HPSEC chromatogram of *G. caudata* polysaccharide shown a single peak at 8.95 ml. The *G. caudata* polysaccharide behaves as a homogeneous system unlike the observed to the polysaccharides from *Botryocladia occidentalis* (Farias, Valente, Pereira, & Mourao, 2000) and *G. cornea* (Melo et al., 2002) red seaweed. Based on Eq. (1) the M_{pk} of PGC is $2.5 \times 10^5\ g\ mol^{-1}$. High molecular mass polysaccharides have been described for other sulfated polysaccharides from seaweeds with values larger than 100 kDa (Lahaye, 2001; Murano et al., 1992; Pomin, 2010; Rodriguez, Matulewicz, Noseda, Ducatti, & Leonardi, 2009; Tashiro, Mochizuki, Ogawa, Mizuno, & Iso, 1996).

The FT-IR spectrum of *G. caudata* from Brazil (data not shown) shows characteristic bands of agar type polysaccharides (1258, 1075, 930 and 891 cm^{-1}) (Christiaen & Bodard, 1983; Maciel et al., 2008; Mollet, Rahaoui, & Lemoine, 1998; Rochas, Lahaye, & Yaphe, 1986; Sekkal & Legrand, 1993). The band at 1258 cm^{-1} correspond to S=O vibration of the sulfate groups (Fournet et al., 1997; Melo et al., 2002; Rochas et al., 1986). As this band possesses a low intensity in PGC it is considered that a small degree of substitution was expected for this polysaccharide. This result was confirmed by the low DS sulfate (0.13) calculated by elemental microanalysis. The absorbance at 930 cm^{-1} has been assigned to the vibration C—O—C bridge in 3,6-anhydro-α-L-galactose (Christiaen & Bodard, 1983) and the skeletal mode of galactans was attributed to the signal at 1075 cm^{-1} (Sekkal & Legrand, 1993).

In agar type polysaccharide the position of sulfate group can be inferred by bands at 800–850 cm^{-1} region. Several reference data shows that bands at 845 and 830 cm^{-1} can be attributed to respectively the 4-*O*-sulfate and 2-*O*-sulfate groups present in D-galactose units, while the signal at 820 and 805 cm^{-1} are due to sulfation on C6 of L-galactose and C2 of the 3,6-anhydro-L-galactose, respectively (Chopin & Whalen, 1993; Lahaye & Yaphe, 1988; Matsuhiro & Rivas, 1993; Mollet et al., 1998; Prado-Fernandez, Rodriguez-Vazquez, Tojo, & Andrade, 2003; Rochas, Lahaye, & Yaphe, 1986; Sekkal & Legrand, 1993; Villanueva, Sousa, Goncalves, Nilsson, & Hilliou, 2010).

G. caudata polysaccharide did not show any signal resolved at 845, 830 and 805 cm^{-1} indicating the absence of sulfation on C-4 and C-2 of D-galactose and on C-2 of 3,6-anhydro-L-galactose residues. Several *Gracilaria* agars were found to possess this sulfation pattern (Mollet et al., 1998; Murano, 1995; Murano et al., 1996; Praiboon, Chirapart, Akakabe, Bhumibhamon, & Kajiwara, 2006; Rochas et al., 1986). The low intense signal at 821 cm^{-1} was assigned to sulfation of C-6 of L-galactosyl residues the biological precursor of the 3,6-anhydro-L-galactose (Mazumder et al., 2002; Rees, 1961).

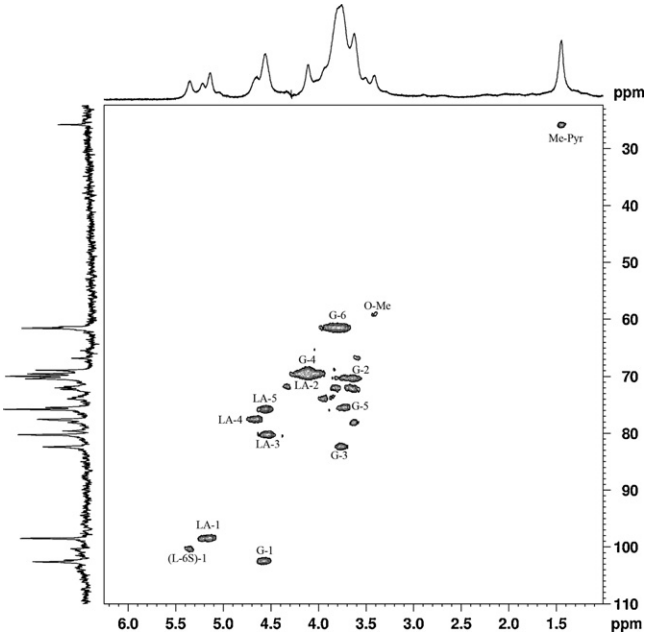

Fig. 2. ^1H and ^{13}C HSQC spectrum of *G. caudata* polysaccharide in D_2O.

Nuclear magnetic resonance (NMR) spectroscopy of ^1H and ^{13}C is an efficient method to analyze the structural features of seaweed polysaccharides (Lahaye, Yaphe, Viet, & Rochas, 1989; Miller & Furneaux, 1997; Usov, Yarotsky, & Shashkov, 1980; Usov, 1984, 1992; Valiente et al., 1992). Fig. 2 shows the two dimensional NMR spectrum of *G. caudata* polysaccharide. The signal from the α anomeric proton at δ 5.13 was assigned to 3,6-α-L-anhydrogalactose (LA) while the signal at δ 4.56 was attributed to β-D-galactose (G) linked to LA (Maciel et al., 2008; Mazumder et al., 2002). No signal was observed at δ 4.43 which was assigned to H-1 of β-D-galactose linked to α-L-galactose-6-sulfate, although the latter residue had been found as a low intense peak in the FT-IR experiment and the signal at δ 5.34 was attributed to anomeric proton of 6-O-sulfate-L-galactopyranose. The intense resonance signal at δ 1.44, attributed to methyl protons of the cyclic pyruvate acetal as 4,6-O-(1-carboxyethylidene) group, together with a signal at δ 5.22, which is described for H-1 of the L-galactose residue linked to a pyruvated D-galactose residue (Izumi, 1973; Mazumder et al., 2002; Murano, 1995; Murano et al., 1992), were found in the ^1H NMR spectral data of *G. caudata* polysaccharide. Pyruvic acid is not frequently described for agarocolloids from *Gracilaria* species (Duckworth, Hong, & Yaphe, 1971; Murano, 1995; Murano et al., 1992). This spectrum also revealed a methylation pattern with resolution of signals at δ 3.41 for protons of methyl groups attached to *O*-6 of β-D-galactose (G6M). The occurrence of methyl ether at C-6 (G) in agars extracted from different *Gracilaria* species has been widely reported, with the degree of substitution varying among species (Craigie, Wen, & VanderMeer, 1984; Duckworth et al., 1971; Furneaux, Miller, & Stevenson, 1990; Ji, Lahaye, & Yaphe, 1985; Lahaye, Rochas, & Yaphe, 1986; Montano et al., 1999; Villanueva & Montano, 1999).

The anomeric region of ^{13}C NMR (δ 90–110) shows two main signals (Fig. 2), which were assigned based on the literature data

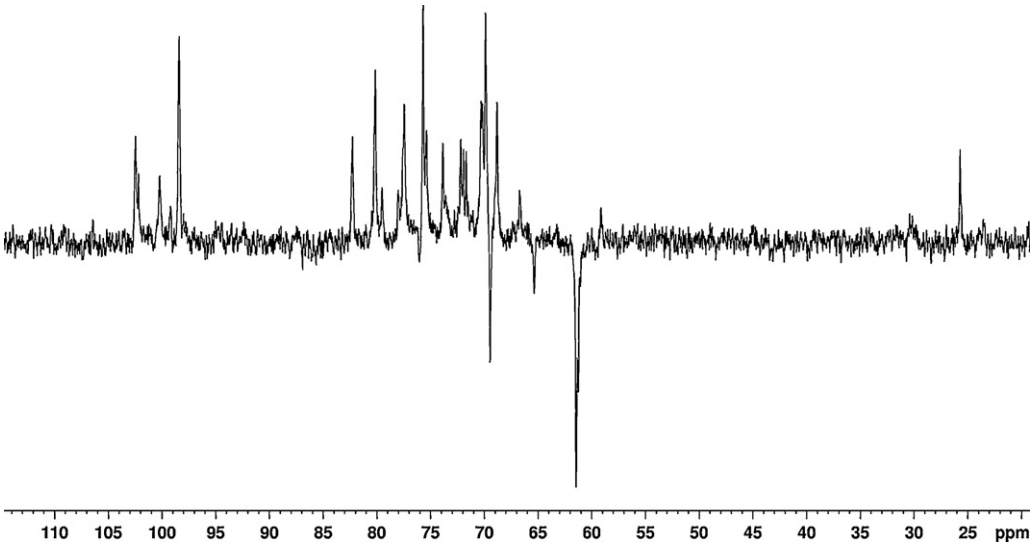

Fig. 3. DEPT 135° spectrum of *G. caudata* polysaccharide.

(Lahaye et al., 1989; Maciel et al., 2008; Miller & Furneaux, 1997; Usov et al., 1980; Valiente et al., 1992). The signals at δ 102.6 and δ 98.5 were attributed to C-1 of β-D-galactose and C-1 of 3,6-α-L-anhydrogalactose, respectively. The small signal at δ 100.3 was assigned to C-1 of α-L-galactose 6-sulfate.

The HSQC spectrum (Fig. 2) shows the correlation of the anomeric carbons with their respective protons at: δ 4.56/102.6 for G residue; δ 5.35/100.3 for L-6S residue and δ 5.13/98.5 for LA residue. The four C-6 atoms at opposite amplitudes in the DEPT spectrum exhibited correlations with their protons at δ 4.10/69.5; δ 3.76/65.4/61.2 and δ 3.78/61.5, respectively for LA (3,6-anhydrogalactose), G (β-D-galactose) and G′ (β-D-galactose linked to α-L-galactose-6-sulfate). Correlations between carbon and proton were also found at δ 3.40/59.1 and δ 1.45/25.7 indicating the presence of amounts of methoxyl and pyruvate groups in the galactose residue. Based on correlations observed in HSQC spectrum and literature data the carbon and hydrogen assignments for *G. caudata* polysaccharide are given in Table 1.

The monosaccharide ratio of polysaccharide from *G. caudata* was estimated from C-1 signal integrals. Therefore, the molar ratio of β-D-galactose (G)/3,6-anhydro-α-L-galactopyranose (LA)/α-L-galactopyranose-6 sulfate unit (L-6S) in *G. caudata* polysaccharide was 1.0:0.8:0.2.

A DEPT 135 experiment was used to investigate the presence of oxymethylene groups in *G. caudata* polysaccharide (Fig. 3), considering that the pulse sequence signals of the carbons bearing two protons have opposite amplitude to the CH and CH_3 carbons. The DEPT spectrum shows signals at δ 69.5, δ 65.4, δ 61.4 and δ 61.2 due to CH_2 protons. The signals at δ 69.5 and δ 61.4 were attributed respectively to C-6 of 3,6-α-L-anhydrogalactose (LA) and β-D-galactose (G) linked, while the signals at δ 65.4 and δ 61.2 have been ascribed for C-6 of α-L-galactose-6-sulfate (L-6S) and β-D-galactose (G′) linked respectively. This spectrum also showed two low intense signals due to CH_3 at δ 59.0 indicating the presence of O-methyl sugar residue in this polysaccharide and at δ 25.7 is attributed to methyl carbon of pyruvate.

Table 1
^{1}H and ^{13}C NMR chemical shifts for *G. caudata* polysaccharide.

Unit[a]	^{1}H chemical shift (ppm)						
	H-1	H-2	H-3	H-4	H-5	H-6	O–Me
G	4.56	3.62	3.75	4.10	3.71	3.78	–
LA	5.13	4.10	4.51	4.66	4.55	4.10	–
L-6S	5.34	[b]	[b]	[b]	[b]	[b]	–
G6M	[b]	[b]	[b]	[b]	[b]	[b]	3.41
Unit[a]	^{13}C chemical shift (ppm)						
	C-1	C-2	C-3	C-4	C-5	C-6	O–Me
G	102.5	70.37	82.3	68.87	75.44	61.5	–
LA	98.5	69.95	80.2	77.5	75.6	69.5	–
L-6S	100.26	[b]	[b]	[b]	[b]	65.4	–
G6M	[b]	[b]	[b]	[b]	73.6	71.8	59.1

[a] Diad nomenclature proposed by Knutsen et al. (1994).
[b] Those signal may be overlapped with signal from unsubstituted units

4. Conclusions

The polysaccharide isolated from *G. caudata* is an agar type polysaccharide composed mainly of by β-D-galactopyranose linked to 3,6-anhydro-α-L-galactose with methyl or pyruvate substituted groups at C-6 of β-D-galactose and with a peak molecular mass of 2.5×10^5 g mol^{-1}. The sulfate content is 1% and the DS sulfate equal to 0.13. The composition suggests a structure very close to the ideal agars.

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