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NMR spectroscopic investigation of agarose oligomers produced by an α-agarase

Cyrille Rochas [a,*], Philippe Potin [b] and Bernard Kloareg [b]

[a] *Centre de Recherches sur le Macromolecules Végétales, CNRS-UPR 5301, BP 53X, F-38041 Grenoble (France)*
[b] *Centre d'Études d'Océanographie et de Biologie Marine, CNRS UPR 4601, BP 74, F-29680 Roscoff (France)*

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ABSTRACT

The ^{13}C NMR signals of various even and odd agarose oligosaccharides with either D-galactose or 3,6-anhydro-α-L-galactose at the reducing end have been assigned. The chemical shifts in water of the agaro- and the neoagaro-oligosaccharides are compared and the influence of dimethyl sulfoxide on the chemical structure of the agaro-oligosaccharides is reported. The 3,6-anhydro-L-galactose residue at the reducing end of agaro-oligosaccharides is in the hydrated form.

INTRODUCTION

Agar is a family of polysaccharides extracted from marine red algae, the Gracilariales and the Gelidiales, consisting of alternating β-(1 → 3)-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose units more or less substituted[1] by sulphate esters, pyruvate acetal, and methyl ethers. An extensive literature[2–4] is devoted to the determination of these structures. The main approach has been ^{13}C NMR analysis of the oligosaccharides obtained upon hydrolysis of agar with β-agarase[4–7]. This enzyme cleaves the β-(1 → 4) linkage between D-galactopyranose and 3,6-anhydro-L-galactose to give a series of a neoagaro-oligosaccharides[8].

Production of oligosaccharides of the agarobiose series, with D-galactose residues at their reducing ends (Scheme 1), involves hydrolysis of agarose with α-agarase, an enzyme which cleaves the α-(1 → 3) linkages between 3,6-anhydro-L-galactose and D-galactose residues. Such oligosaccharides were first described by Yaphe and co-workers[9–11] in their investigation of agar degradation by the bacterial strain GJ1B. ^{13}C NMR spectra were reported[10] for an unfractioned mixture of the

* Corresponding author (present address): Laboratoire de Spectrométrie Physique (Associé au CNRS), Université Joseph Fourier, BP 87, F-38402 Saint Martin d'Hères, France.

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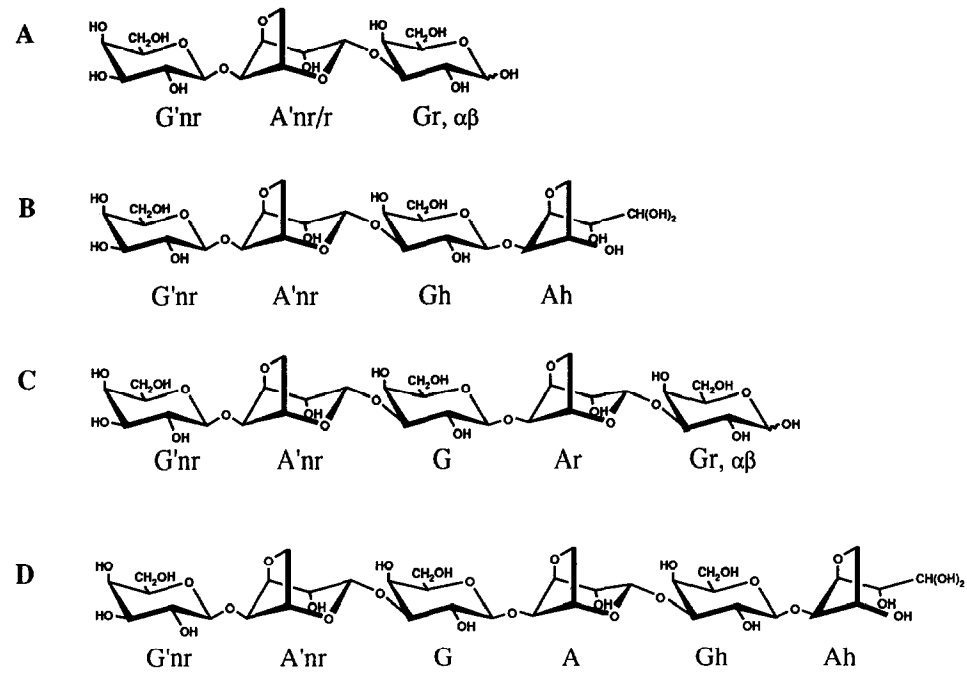


Scheme 1. Structure of the oligosaccharides released by the action of agarolytic enzymes of *Alteromonas agarlyticus*[12] on agarose. **A**: agarotriose; **B**: agarotetraose; **C**: agaropentaose; **D**: agarohexaose.

agaro-oligosaccharides produced by the partially purified α-agarase of the bacterium.

Recently, we identified strain GJ1B as *Alteromonas agarlyticus*, achieved complete purification of the α-agarase, and described in more detail the agarolytic system of this bacterium[12]. It consists of two enzymes, α-agarase and a β-galactosidase specific for the presence of the 3,6-anhydro-L-galactose units at the reducing ends of the reaction products. β-Agarase activity was not detected. Depending on the extent of purification of the α-agarase, either even or both even and odd agaro-oligosaccharides are released upon hydrolysis of agarose. Agaro-oligosaccharides with an even number of monosaccharides units are produced when agarose is incubated with the α-agarase purified to homogeneity, whereas mixtures of even and odd agaro-oligosaccharides are produced when the polymer is hydrolysed with partially purified α-agarase fractions, which also show β-galactosidase activity[12]. We now report on the characterisation of both oligosaccharide series by ^{13}C and ^{1}H NMR spectroscopy.

EXPERIMENTAL

Agarose was kindly provided by Hispanagar (Burgos, Spain). Neoagaro-oligosaccharides were obtained by hydrolysis of agarose with β-agarase, as described

previously[7]. Agarotetraose and agarohexaose were prepared using the purified α-agarase fraction from *Alteromonas agarlyticus*, whereas agarotriose and agaropentaose were prepared with the partially-purified, using affinity-chromatography, α-agarase fraction[12]. The various agaro-oligosaccharides were fractionated using gel filtration as previously described.

NMR spectra of 1–5% (w/v) solutions of oligosaccharides were recorded at room temperature on a Bruker AC 300 instrument. For the ^{13}C spectra, a spectral width of 15 kHz, acquisition delays of 0.27–0.54 s, and 8k or 16k data points were used. In order to increase the signal resolution, a sine-bell function was used before the Fourier-transformation and the data were zero-filled to 16k or 32k. For a better visualisation of the carbonyl carbon resonances, a relaxation delay of 3–8 s was used. The 2D carbon–proton correlation and the distortionless enhancement polarisation transfer (DEPT) spectra were determined using the programs provided by Bruker. For the ^{1}H spectra, a spectral width of 3 kHz and an acquisition delay of 1–6 s were used.

Infrared spectra of oligosaccharides (2–4% w/w) were recorded in a KBr pellet, using a 1720X Perkin–Elmer FT IR spectrometer. The fast atom bombardment (FAB) mass spectra were obtained using a glycerol matrix with a Model 2000 Nermag R 1010C mass spectrometer equipped with a M-Scan Wallis-type gun.

RESULTS AND DISCUSSION

Nomenclature.—The nomenclature (Scheme 1) used for the various agaro-oligomers released by the agarases of *Alteromonas agarlyticus* is that previously developed for oligogalactans[7,13–15]. Briefly, **G** and **A** represent the *O*-linked β-D-galactopyranose and the 4-*O*-linked 3,6-anhydro-α-L-galactopyranose, respectively. The letters **nr** and **r** refer to galactose residue close to the nonreducing end and to the reducing end, respectively, and the internal units are not labelled. In agarotriose, because of the median position of the anhydro sugar, this sugar can be labelled either **nr** or **r**. The oligomers having either unit **A** or unit **G** at the nonreducing end are currently named neoagaro- or agaro-oligosaccharides in the literature. To take into account some possible minor differences between the neoagaro- and the agaro-oligosaccharides the residues towards the nonreducing end of the agaro-oligosaccharides are labelled **G'nr** and **A'nr**. The presence of the hydrated aldehydic form of unit **A** at the reducing end is noted by the letter **h** instead of **r** (Scheme 1).

Odd oligosaccharides.—Given the chemical shifts of α,β-D-galactose[16] and of neoagaro-oligosaccharides[6,7] assignment of the ^{13}C NMR signals of the odd agaro-oligosaccharides was straightforward for units **G**, **Ar**, **Grα**, and **Grβ** (Table I). Results are very consistent with those previously published for the neoagarose series[6,7], with the exception of the lack of any signal for 3,6-anhydro-galactose at the nonreducing end[9]. Depending on signal acquisition and treatment, C-1 and C-2 of **A'nr/r** were split into two close signals, due to the influence of the anomeric

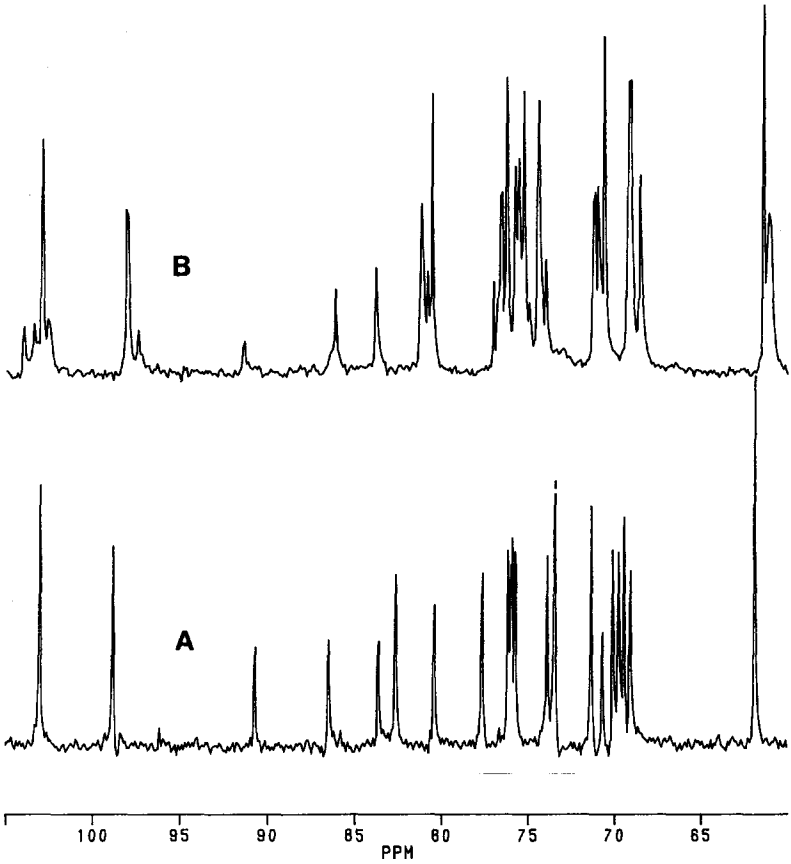


Fig. 1. ^{13}C NMR spectra of agarotetraose: A, in water; B, in Me_2SO.

forms of **Gr.** As for the neoagarose series, the chemical shifts of carbons of unit **A'nr** and **Ar** were identical. This probably reflects the rigidity of the pyranose ring of the 3,6-anhydro-α-L-galactopyranose. In addition, using quantitative analysis conditions, the integral of all the carbons was in excellent agreement with the tri- and penta-saccharide structures.

Even oligosaccharides.—The ^{13}C NMR spectrum of the tetrasaccharide and that of the hexasaccharide presented two major differences compared with the spectra of the odd agaro-oligomers and neoagaro-oligosaccharides. The absence of a C-1 signal for the α or β configuration was observed and a resonance occurred at an unusual position[17] for an oligosaccharide (90.72 ppm). The spectrum of the tetrasaccharide presented 22 signals (Fig. 1); two signals (73.47 and 61.88 ppm) were broad and resolution-enhancement methods showed the almost superposition of two peaks. In addition, the integral of these broad peaks was higher than that of the others signals. Overall, the number of observed signals (22 + 2) corresponded with the number of signals for a tetrasaccharide and the reducing sugar assay[18],

TABLE I

^{13}C NMR chemical shifts (ppm) of the trisaccharide and of the pentasaccharide produced by the enzymic hydrolysis of agarose by the partially purified α-agarase of *Alteromonas agarlyticus* (affinity chromatography fraction[12])

Sugar	C-1	C-2	C-3	C-4	C-5	C-6
G'nr	102.98	71.34	73.47	69.45	76.19	61.88
G	102.74	70.59	82.54	69.12	75.79	61.79
Grβ	97.11	71.86	83.00	69.23	75.60	61.79
Grα	93.12	68.39	79.81	69.89	71.01	61.99
A'nr [a]	98.83 [b]	70.14 [b]	80.44	77.69	75.92	69.79
Ar [a]	98.83	70.14	80.44	77.67	75.92	69.79

[a] For the trisaccharide, the carbons of the anhydrogalactose unit can be labelled either "**nr**" or "**r**".
[b] Shoulder due to **Grα** and **Grβ**

used with neoagarotetraose as standard, confirmed the oligomer as a tetrasaccharide. In this respect, it is noteworthy that both sugars behaved comparatively, their colorimetric responses in the range 200–700 $\mu g\ mL^{-1}$, differing by less than ±4%. Yet, as suggested previously[12], agaro-oligosaccharides were not detectable in the reducing-sugar assay below a threshold concentration of 30 $\mu g\ mL^{-1}$.

Based on their similarity with the chemicals shifts of the odd agaro-oligosaccharides (Table I) and neoagaro-oligosaccharides[6], eighteen signals were unequivocally assigned to the units **G'nr**, **Gh**, and **A'nr** (Table II). Only three chemical shifts (103.04, 70.74, and 82.64 ppm) of an internal galactose unit differed slightly from the expected values (102.74, 70.59, and 82.54 ppm) for a true **G** unit (Tables I and II). These differences suggest that, as postulated by Yaphe and co-workers[9–11], the reducing end and neighbouring sugar of this 3-linked galactose unit is the open, hydrated aldehydic form of the 3,6-anhydro-L-galactose residue (**Ah**) at the reducing end. This hypothesis is supported by the presence of the unusual but characteristic chemical shift at 90.72 ppm of the hydrated form of the aldehyde[19]. The presence of this form was also deduced from the variations of the chemical shift of C-1, C-2 and C-3 signals of the **Gh** unit (63.69, 71.79, and 82.59 ppm respectively)

TABLE II

^{13}C NMR chemical shifts (ppm) of the tetrasaccharide and of the hexasaccharide produced by the enzymic hydrolysis of agarose by the purified α-agarase of *Alteromonas agarlyticus*[12]

Sugar	C-1	C-2	C-3	C-4	C-5	C-6
G'nr	102.98	71.34	73.47 [a]	69.45	76.19	61.88 [b]
G	102.70	70.60	82.58	69.11	75.78	61.80
Gh	103.04	70.74	82.64	69.11	75.78	61.88 [b]
A'nr	98.82	70.12	80.44	77.68	75.94	69.77
A	98.82	70.12	80.44	77.62	75.94	69.77
Ah	90.72	73.47 [a]	83.68	86.52	76.03	73.89

[a,b] Overlapping

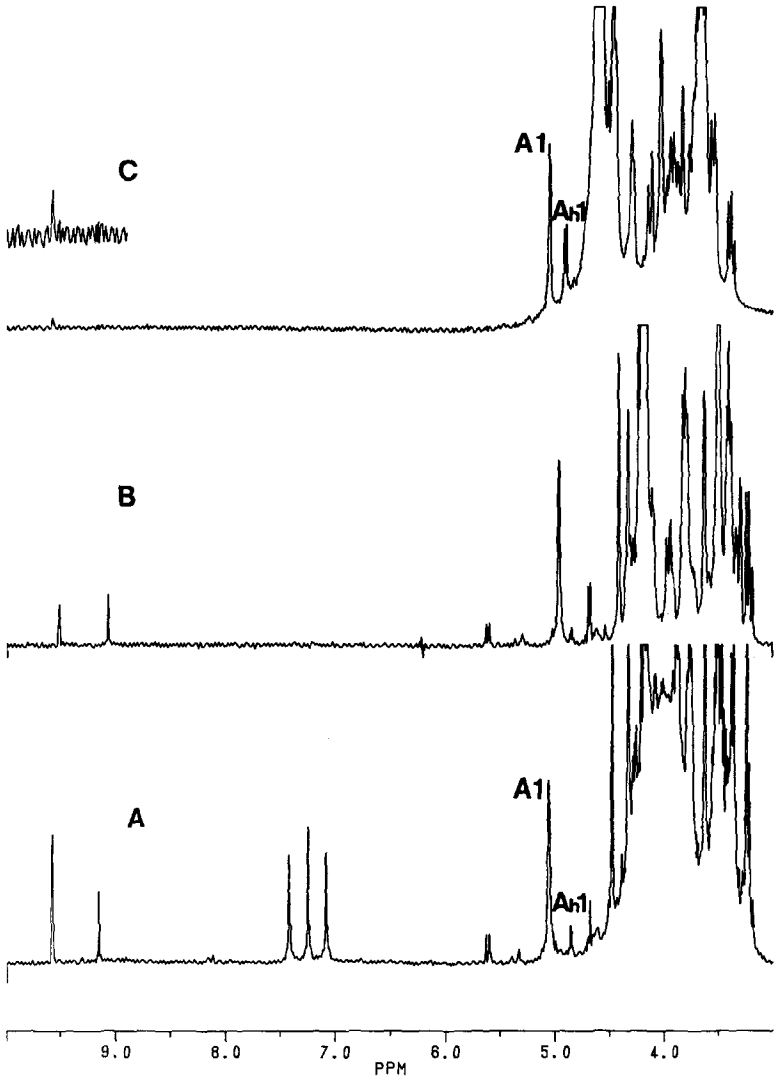


Fig. 2. ^{1}H NMR spectra of agarotetraose: A, in Me_2SO; B, in 7:2 Me_2SO–water; C, in water.

in the ^{13}C NMR spectrum of agarotetraitol, obtained after reduction[20] of agarotetraose. These new chemical shifts were in agreement with values reported by Usov et al.[21] and Miller et al.[22] for agarobiitol.

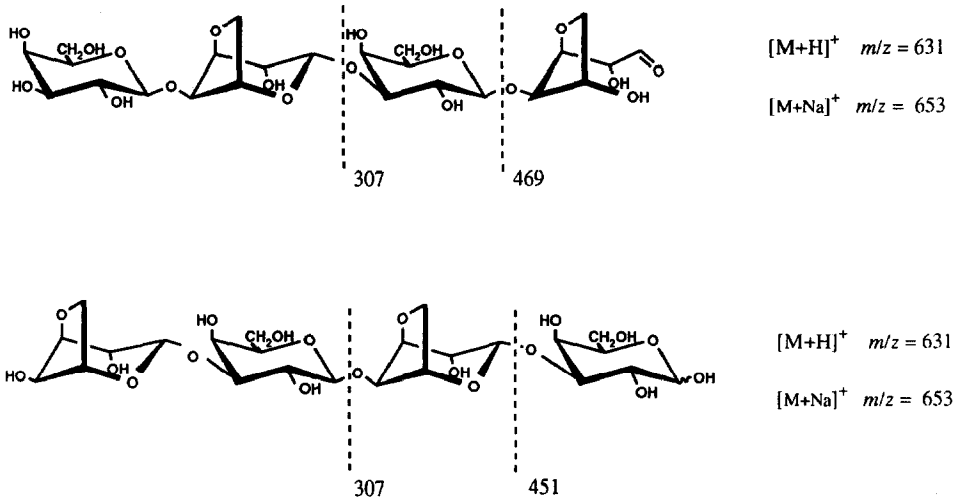
The ^{1}H NMR spectrum in water showed, however, that the chemical equilibrium between the aldehydic and the hydrated aldehydic form of the 3,6-anhydrogalactose was not totally shifted toward the hydrated aldehydic form. In addition to the H-1 signal at 4.93 ppm, characteristic of the latter form, a small typical signal was observed at 9.53 ppm for the aldehyde (Fig. 2). Surprisingly, we also noticed that the signal intensity of carbons or protons of the **Ah** unit were

slightly lower (70%) than those of the **G'nr**, **G**, **Gh**, **A'nr** or **A** units. This difference was independent of the experimental conditions: acquisition time, temperature, solution in 4 M urea. So far we can provide no consistent explanation for this finding.

Signal assignment from the spectrum (not shown) of agarohexaose (Table II) was logically derived from that of the tetrasaccharide. The signal intensity increase for the carbons of the **A'nr** unit was due to the superposition of those from the **A** unit. As previously noted for the odd agaro-oligosaccharides (see above) and for the neoagaro-oligosaccharides[6,7], the chemical shift of carbons of units **A**, **Ar**, and **Anr** were identical. The chemical shifts of the **G** unit (Table II) were in close agreement with previous data[6,7].

Mass spectrometry and ^1H NMR spectroscopy in Me_2SO.—The ^1H and ^{13}C NMR spectra of the agaro-tetrasaccharide in Me_2SO differed markedly from those recorded in water (Figs. 1 and 2). Firstly, carbonyl signals were detected at 204.38 and 190.72 ppm and were correlated (2D C–H correlation NMR spectroscopy) with protons observed at 9.59 and 9.15 ppm, respectively, in the ^1H spectrum (Fig. 2). Gradual addition of water to a Me_2SO agarotetrasaccharide solution resulted in the gradual disappearance of the proton signals near 9 ppm (Fig. 2). This suggest that, in Me_2SO both the aldehydic and the hydrated aldehydic forms of agarotetraose are present, whereas the equilibrium is shifted towards the hydrated form in the presence of water. The addition of water to Me_2SO (Fig. 2) or of Me_2SO to water changed this equilibrium. Secondly, in comparison with the agarotetraose spectrum recorded in water, in Me_2SO many other new signals appeared both in the ^1H and the ^{13}C spectra (Figs. 1 and 2). The signals for C-1 and C-6 of the **G** unit were split into many signals (103.81, 103.18, 102.72, and 102.34 and 61.12, 60.86, and 60.78 ppm, respectively); that of C-1 of the **A'nr** unit was split into only two signals (97.82 and 97.15 ppm) and a DEPT spectrum showed a splitting for C-6 of the **Ah** unit (74.00 and 73.76 ppm) but not for C-6 of the **A'nr** unit. Due to the complexity of the spectrum, it was impossible to verify whether signal splitting occurred for all the signals of the different units. Formation of a certain proportion of different hemiacetals between the different hydroxyls of **Ah** and **Gh** units would probably explain the presence of these new signals. This possibility is supported by the drastic conformational change of the sugar when Me_2SO is replaced by water as revealed by a spin–spin coupling of H-1 of unit **Ah** of 2.2 Hz in Me_2SO and 6.3 Hz in water, at 4.85 and 4.93 ppm, respectively.

Analysis of dried agaro- and neoagaro-tetraose by positive-ion FAB mass spectroscopy (Scheme 2) confirmed their structural differences as well as their degree of polymerisation. In addition, as shown by presence of the quasi-molecular ion $[M + H]^+$ at m/z 631, FABMS indicates that, in the solid state, the aldehydic form is predominant in agarotetraose. A major difference between the two tetrasaccharides was observed in the cleavage of the glycosidic linkages, according the classical pathway A fragmentation common to sugars[23]. Ions at m/z 307 and



Scheme 2. Fragmentations observed in positive-ion FAB mass spectrometry of agaro- and neoagaro-tetraose.

469 were recorded for agarotetraoase whereas ions at m/z 307 and 451 were recorded for neoagarotetraose. Another significant difference was the small amount of ion $[M + H - H_2O]^+$ at m/z 613 for agarotetraose compared to neoagarotetraose. Predominance of the aldehydic form in the solid state was confirmed by the carbonyl band at 1728 cm^{-1} in the infrared spectrum of agarotetraose.

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