The Chemical Structure of *Gracilaria crassissima* (P. *et* H. Crouan in Schramm *et* Mazé) P. *et* H. Crouan in Schramm *et* Mazé and *G. tikvahiae* McLachlan (Gigartinales, Rhodophyta) Cell-Wall Polysaccharides¹

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Abstract

The matricial and part of the fibrillar polysaccharides from the cell walls of *Gracilaria crassissima* and *G. tikvahiae* were investigated using a sequential solvent extraction, ¹³C, ¹H nuclear magnetic resonance and infrared spectroscopy, anion exchange fractionation, and X-ray diffractometry. The gel strength of agar fractions from *G. crassissima* were also measured.

Agar from *G. crassissima*, composed essentially of agarobiose repeating units with its biological precursor, was extracted with cold water and boiling 60% ethanol. The content of 6-*O*-methylated agarobiose repeating units in this agar was low, and the substituted disaccharide was principally concentrated in one highly methylated fraction extracted from the alga with boiling 80% ethanol. Alkali treatment of fractions converted the mainly charged molecules to neutral agarose that eluted essentially with distilled water from the anion-exchanger. The gel strength, 3,6-anhydrogalactose and sulfate contents of alkali-treated fractions were comparable or better than commercial agar or agarose. Thus, *G. crassissima* represents an excellent potential commercial resource for agar and agarose.

In contrast, with *G. tikvahiae* the 6-*O*-methylated agar-containing 4-*O*-methyl *L*-galactode, was the principal extract in water at 121 °C, probably reflecting cross-linkages of cell-wall polymers. Alkali treatment of this fraction did not affect its elution pattern through DEAE-Sephadex, demonstrating that the charge on this agar was not associated with biological precursor repeating units. Analysis of the ¹³C NMR spectra of the polymers revealed resonances that were assigned to the 4-*O*-methyl α -*L*-galactopyranose present in this agar.

Anion exchange chromatography of agar fractions from these two algae and the different gel strengths obtained between alkali-treated fractions from *G. crassissima* indicated that the extracts obtained by the

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sequential solvent method were heterogeneous. A combination of sequential solvent extraction of agar and anion exchange chromatography is proposed for obtaining better chemically defined agaroses.

Finally, X-ray diffractometry definitively demonstrated the presence of cellulose in the fibrous material recovered after extraction of agar from these two algae.

Introduction

The cell-wall of marine red algae (Rhodophyta) has been described as a reticulate network of fibrils embedded in amorphous matricial polysaccharides (Cronshaw *et al.* 1958, Myers and Preston 1959a, 1959b, Dawes *et al.* 1961). The chemical structure of the matricial polysaccharides synthesized by species of *Gracilaria* is under investigation in many laboratories as they represent important resources for the commercially valuable gelling polysaccharides, agar (McLachlan 1985). The latter is a family of molecules, or agaroses, built on a disaccharide repeating unit referred to as agarobiose, and composed of (1,4) linked β -D-galactose and (1,3) linked 3,6-anhydro- α -L-galactose (Araki 1966, Fig. 1). The physical and rheological properties of agaroses depend on the presence and concentration of substituent groups, such as methyl ethers, sulfate esters and pyruvate ketals (Araki 1966), on the repeating unit (Guiseley 1970, Arnott *et al.* 1974, Dea and Rees 1987). A biological precursor of the agarobiose repeating unit has been recognized in the disaccharide having the 3,6-anhydro-L-galactose residue replaced by L-galactose-6-sul-

fate as an algal enzyme or an alkali treatment converts the sulfated sugar into the anhydride residue (Rees 1961 a, 1961 b).

The chemical heterogeneity of agaroses is now well established and is best described by anion-exchange fractionation and solvent extraction experiments (Duckworth and Yaphe 1971, Izumi 1970, 1971, 1972, Lahaye and Yaphe 1988, Lahaye *et al.* 1986). Within a species, this heterogeneity depends mainly on tissue types and growth conditions (Craigie and Wen 1984, Christiaen *et al.* 1987, Lahaye and Yaphe 1988).

In the present work, chemical properties determined by nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and anion-exchange chromatography, as well as gel strength of agarose fractions synthesized by *Gracilaria crassissima* (P. et H. Crouan in Schramm *et Mazé*) P. et H. Crouan in Schramm *et Mazé*, are reported. Because 4-O-methyl-L-galactose has been reported in agars (Araki *et al.* 1967, Bird *et al.* 1987, Craigie and Wen 1984, Duckworth *et al.* 1971, Hirase *et al.* 1983, Ji *et al.* unpublished, Lahaye 1986), we also extracted sequentially with the solvent method and fractionated on DEAE-Sephadex A 50 the 4-O-methyl galactose containing agar from *Gracilaria tikvahiae* MacLachlan (Craigie and Wen 1984, Duckworth *et al.* 1971) in order to determine typical ^{13}C NMR chemical shifts associated with this structure, thus facilitating its detection in agars from other species. Finally, part of the insoluble residue recovered after the sequential agar extraction has been identified by X-ray diffractometry as cellulose, thus proving the existence of this structural polysaccharide in the Gracilariaceae.

Material and Methods

Gracilaria crassissima (P. et H. Crouan in Schramm *et Mazé*) P. et H. Crouan in Schramm *et Mazé* was collected at Point Sable, St Lucia, in 1983 (NRCC voucher No. 9138) and *Gracilaria tikvahiae* McLachlan was the same sample referred to as *G. foliifera* in Duckworth *et al.* (1971). The voucher specimen is deposited with the National Research Council (NRCC), Halifax, Canada B3H 3Z1. The air dried algae was treated, and their agar extracted and alkali-modified according to the procedures described by Lahaye *et al.* (1986).

Fig. 1. Structure of:
A/agarobiose and substituted agarobiose repeating units,
R = H: agarobiose
R = CH₃: 6-O-methylated agarobiose
R = 4-O-methyl α -L-galactose (proposed structure Craigie and Wen 1984, Hirase *et al.* 1983)
B/biological precursor to the agarobiose repeating unit

The major agar fractions were chromatographed on DEAE-Sephadex A 50 according to the procedure of Duckworth *et al.* (1971) described in Ji *et al.* (1985).

^{13}C and ^1H nuclear magnetic resonance (NMR) spectra were recorded at 80 °C as described by Lahaye *et al.* (1986). The ^{13}C NMR chemical shifts are expressed in parts per million (ppm) relative to internal dimethylsulfoxide (DMSO) and converted to values related to external tetramethylsilane (TMS, conversion constant 39.6). The ^1H NMR chemical shifts were measured in ppm from external sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS). A Bruker WH 400 operating at 100.6 and 400.1 MHz was used to record ^{13}C and ^1H NMR spectra, respectively.

Infrared spectra of agar films were recorded on a Perkin-Elmer 297 spectrophotometer. The films were made and ratio of IR absorbances were calculated according to the method of Rochas *et al.* (1986 a).

Gel strength of agar fractions were measured using the semimicro-gelometer of Goring (1956) as described by Lahaye *et al.* (1988).

Insoluble residue for X-ray diffraction analysis were obtained as follows. The algal residues after the sequential solvent extraction were stirred in 1.5N KOH (100 ml, 4 h) at room temperature, neutralized with HCl, briefly washed with distilled water on glass fiber pads in a Buchner funnel using suction, boiled six times (0.5 h) in distilled water (100 ml) containing acetic acid (5 ml) and sodium hypochlorite (0.5 ml of a 100% solution), washed with acetone, air dried and freeze-dried. X-ray diffraction patterns were performed on and recorded on a flat film in a Wharms camera. A Philips X-ray unit operating at 40 kV and 20 mA was used with a Cu-tube as a radiation source. The Cu-K $_2$ radiation ($\lambda = 1.541 \text{ \AA}$) was monochromatized by means of a Ni-filter. The interplanar spacings were calculated with the help of NaF powder deposited on the sample as calibration standard.

Results and Discussion

Extraction of agar polymers

The extraction of agar from *G. crassissima* with water at room temperature (CX) and with boiling 60% ethanol (Fig. 2) was suggestive of loosely associated molecules (Lahaye *et al.* 1986, Lahaye and Yaphe 1988), and an overall yield of 45% of polymers was obtained on the basis of the starting algal dry weight. In contrast, drastic conditions were necessary to extract *G. tikvahiae* agar (water at 121 °C, Fig. 2) although the 6-*O*-methylated agarobiose repeating units

Fig. 2. Yield of agar polymers extracted from *Gracilaria crassissima* and *G. tikvahiae* with water at 22 °C (CX), boiling 80, 60, 40, 20% ethanol-water mixtures, and water at 100 °C (0) and 121 °C (Au).

and 4-*O*-methyl-*L*-galactose present (Craigie and Wen 1984, Duckworth *et al.* 1971) should have increased its solubility in alcohol rich solvents (Lahaye *et al.* 1986). A lower overall yield of polymers (26.1% on the basis of the starting dry weight of alga) was also obtained compared with that of *G. crassissima*. The high energy required for the extraction of this methylated agar most probably reflected the presence of cross-linkages between agar and other cell-wall polymers. Cross-linkages between cell-wall polymers are known to exist in land plants (Fry 1986), but further work is required to clearly establish their existence and to determine their nature in marine red algae.

Anion exchange chromatography

The relative concentration of neutral and charged polysaccharides in an agar can be estimated from its anion-exchange chromatogram (Duckworth and Yaphe 1971, Duckworth *et al.* 1971, Izumi 1970, 1971, 1972, Ji *et al.* 1985, Lahaye 1986). The DEAE-Sephadex A 50 elution patterns of the native cold water (CX), 60 and 40% ethanol extracts from *G. crassissima* and autoclaved extract from *G. tikvahiae* established that these algae synthesized agars composed preponderantly of charged polymers as the latter were essentially recovered in the 0.5 and 1.0 M NaCl fractions (Figs 3 and 4). The differences noted between the fractionation patterns of native and alkali-treated *G. crassissima* extracts (Fig. 3) were also observed with agars from other species (Young 1974, Usov *et*

Fig. 3. DEAE-Sephadex A 50 fractionation pattern of native and alkali-treated cold-water (CX, 22 °C), boiling 60 and 40% ethanol extracts from *Gracilaria crassissima*. The yields of polymers eluted with distilled water (0), 0.5, 1.0 and 2.5 M NaCl solutions, and recovered with 6.0 M urea from the Sephadex matrix, represent percentages of total carbohydrate applied to the union exchanger.

al. 1979, Friedlander *et al.* 1981, Lahaye 1986) and reflected the decrease in charge density of the polymers, attributed principally to the conversion by alkali of *L*-galactose-6-sulfate to 3,6-anhydro-*L*-galactose. In contrast, alkali treatment did not markedly affect the elution pattern of the *G. tikvahiae* autoclaved fraction (Fig. 4), indicating that other charged groups were present in this agar. The slight decrease in the yield of neutral polysaccharides recovered after alkali treatment of that fraction may be related to the increase in the 6.0 M urea fraction eluted from the anion exchange column. The latter fraction consisted of low charged, methylated agaroses in other *Gracilaria* agars

Fig. 4. DEAE-Sephadex A 50 fractionation pattern of native and alkali-treated autoclave extract from *Gracilaria tikvahiae* (see Fig. 3 for legend).

(Ji *et al.* 1985) and may be retain on the DEAE-Sephadex matrix because of some affinity with the dextran. The elution pattern of the native autoclaved fraction differed from that published by Duckworth *et al.* (1971). Possibly these polymers did not represent all the polysaccharides synthesized by *G. tikvahiae*. Lastly, the DEAE-Sephadex A-50 elution patterns (Figs 3 and 4) demonstrated that these extracts were not homogeneous because each contained neutral and charged molecules. A method using both the sequential solvent extraction of agar and fractionation by anion-exchange chromatography should permit agarose molecules of greater chemical homogeneity to be obtained.

Infrared spectroscopy

Infrared spectroscopy is a convenient method to follow sulfate and 3,6-anhydrogalactose concentrations in agars (Lahaye 1986, Lahaye and Yaphe 1988, Rochas *et al.* 1986 a). The spectra of fractions from both algae had absorbances typical of agar (Christiaen and Bodard 1983, Lahaye and Yaphe 1988, Rochas *et al.* 1986 a). The spectra of agar extracted from *G. crassissima* had a strong absorbance at 930 cm^{-1} attributed to 3,6-anhydrogalactose (Stanley 1963) and a weak absorbance for total sulfate at 1250 cm^{-1} (Lloyd *et al.* 1961). The ratio relating 3,6-anhydrogalactose (930 cm^{-1}) and total sulfate (1250 cm^{-1}) to sugar content (2920 cm^{-1} , Rochas *et al.* 1986 a) are reported in Table I. For comparison, values for a commercial agarose sample (Seakem, lot #711801, Marine Colloids FMC Corp.) are also reported. Alkali-treated cold water, 60 and 40% ethanol extracts had ratios of absorbances indicating similar concentrations of

Table I: Gel strength and ratio of infrared absorbances relating 3,6-anhydrogalactose (3,6AG, 930 cm^{-1}) and total sulfate (1250 cm^{-1}) to total sugar content (2920 cm^{-1}) of agar fractions from *Gracilaria crassissima*, commercial agar and agarose.

Sample	3,6AG (930/2920)	Sulfate (1250/2920)	Gel strength N/cm ²
Agarose	1.612	0.115	3423.7
Agar			2099.3
<i>G. crassissima</i> alkali-treated fractions			
22 °C water	1.542	0.113	2060.1
60% ethanol	1.457	0.117	2099.3
40% ethanol	1.507	0.115	4365.5
20% ethanol	1.068	0.243	
100 °C water	1.186	0.120	
121 °C water	1.274	0.120	
native fraction			
22 °C water	0.967	1.066	

3,6-anhydrogalactose to that of agarose. A high ratio relating total sulfate to total sugar content (1250/2920 cm^{-1}) was obtained from native cold water, alkali-treated 20% ethanol and hot water (100 °C) extracts (Table I). Other agar fractions showed ratios for sulfate content close to that of agarose (Table I).

As previously observed (Lahaye and Yaphe 1988), alkali treatment of the cold water extract greatly decreased the sulfate and increased the 3,6-anhydrogalactose contents since marked changes were observed in their respective IR ratio of absorbances (Table I). Furthermore, the absorbance at 820 cm^{-1} attributed to galactose-6-sulfate (Stancioff and Stanley 1969) observed on the IR spectrum of the native cold water extract was absent on the spectrum of the alkali-treated fraction.

The variations of total sulfate and 3,6-anhydrogalactose contents in alkali-treated fractions from *G. tikvahiae* are shown on Figure 5. Values for 3,6-anhydrogalactose lower than 1 indicate a reduced concentration of anhydro-sugar in these fractions than in agarose; values for sulfate greater than 1 indicate a higher concentration of sulfate than in agarose. In agreement with previous observations (Lahaye *et al.* 1986), the sulfate content was highest in the boiling 20% ethanol and boiling water fractions and that of the 3,6-anhydrogalactose lowest in the autoclaved extract. In contrast, a lower concentration of sulfate was also observed in the latter fraction. Values of 40.8 to 47.5% and about 1.2% for 3,6-anhydrogalactose and sulfate concentrations, respectively, have been reported for this agar (Craigie and Wen 1984, Duckworth *et al.* 1971).

Fig. 5. Relative concentration of 3,6-anhydrogalactose (3,6-AG) and sulfate in alkali-treated agar extracted from *Gracilaria tikvahiae* with boiling 60, 40, 20% ethanol-water solutions and water at 100 °C (0) and 121 °C (Au). The concentrations are in arbitrary units calculated from infrared absorbance ratios (see text for details).

¹³C and ¹H NMR spectroscopy

NMR spectroscopy demonstrated that the agarose from *G. crassissima* was composed of agarobiose, 6-*O*-methylated agarobiose and agarobiose precursor repeat units. Indeed, the ¹³C NMR spectra of the cold water (22 °C), 60 and 40% ethanol extracts from *G. crassissima* showed 12 major signals assigned to the carbons of the agarobiose repeat unit (Fig. 6, Usov *et al.* 1980, Rochas *et al.* 1986 b); that of the 80% ethanol extract showed typical signals assigned to polymers composed essentially of 6-*O*-methylated agarobiose repeat units (Fig. 6, Nicolaissen *et al.* 1980, Lahaye 1986). Signals for disaccharide repeat units, having *L*-galactose-6-sulfate replacing 3,6-anhydro-*L*-galactose, were observed on the spectrum of the cold water and 60% ethanol extracts and barely exceeded the noise level on the spectrum of the 40% ethanol extract (Fig. 6). On alkali treatment of the polymers, the signals assigned to disaccharides containing *L*-galactose-6-sulfate disappeared, and spectra typical of agarose were obtained (cf Fig. 7).

Similarly, resonances for protons of the agarobiose repeat unit (A, G; Welti 1977) and 6-*O*-methylated agarobiose repeat unit (Lahaye 1986) were observed on the ¹H NMR spectra of native 80% ethanol, native and alkali-treated cold water (CX), 60, 40, 20 ethanol, and hot water (100 °C, 121 °C) extracts (Table II, Fig. 7). The degree of substitution with methoxyl groups, *i.e.* the number of methoxyl groups per disaccharide repeat unit (DS, Fig. 8) was calculated from the integral of the methyl protons (3.41 ppm) and G1 (integral of Me/3 / (G1 + A5 + A3 - A1 - A4)) on the spectra of alkali-treated polymers, except that of the

Fig. 6. ^{13}C NMR spectra of *Gracilaria crassissima* agar extracted with cold-water (CX), boiling 80, 60 and 40% ethanol-water solutions. A and G refer to carbons of the anhydrogalactose and galactose residue, respectively, in the agarobiose repeat unit; A* and G* to carbons of L-galactose-6-sulfate and galactose, respectively, in the biological precursor to the agarobiose repeat unit, and G' and Me to carbons of the 6-O-methylated galactose and the methyl group, respectively, in the 6-methylated agarobiose repeating unit. Between 2000 and 4000 transients were recorded for these spectra with a spectral window of 12.5 KHz, a relaxation delay of 0.5 s and an acquisition time of 0.655 s.

80% ethanol extract. Additional signals observed on the spectrum of native polymers were assigned to disaccharide repeat units having α -L-galactose-6-sulfate or α -L-galactose instead of 3,6-anhydro- α -L-galactose (Fig. 6, Lahaye 1986). The integral of the signals at 5.28 ppm assigned to H-1 of 3,6-anhydro- α -L-galactose (A1) were used to calculate the percentage of L-galactose-6-sulfate and L-galactose in the polymers (Fig. 8, integral of $A^*1 \times 100 / A1 + A^*1$). There was a low concentration of methoxyl (DS 0.03) and a high concentration of L-galactose-6-sulfate and α -L-galactose (36%) in the cold water extract. In contrast, agar extracted with boiling 80% ethanol had a high methoxyl (DS 0.84) and a low L-galactose-6-sulfate and α -L-galactose (2%) content. The degree of substitution with O-methyl

Fig. 7. ^1H NMR spectra of *Gracilaria crassissima* agar fractions: A/alkali-treated cold-water; B/native 80% ethanol; C/native cold-water extracts.

G, G', G'' and A, A', A'' refer to protons of the galactose and 3,6-anhydrogalactose residues, respectively, in the agarobiose repeating unit; 6-Me refers to the methyl protons in the 6-methylated agarobiose repeating unit; A*1 refers to proton 1 of α -L-galactose-6-sulfate in the biological precursor to agarobiose.

groups and the L-galactose-6-sulfate and α -L-galactose content decreased from the 60% ethanol extract (DS 0.16 and 22%, respectively) to the 40% ethanol extract (DS 0.06 and 11%, respectively) and increased in the 20% ethanol and hot water (100 °C, 121 °C) extracts (DS 0.08–0.013 and 18–27%, respectively, Fig. 7). The signal assigned to α -L-galactose 6-sulfate and α -L-galactose disappeared on alkali treatment of all the *G. crassissima* agar fractions, suggesting that it is most likely associated with the agarobiose precursor repeating unit.

The minor concentration of highly methylated agar (6-O-methylated agarose) obtained in the 80% ethanol extract from *G. crassissima* (Figs 5, 7) confirmed previous results demonstrating that methylated agars were soluble in high concentrations of hot ethanol-water solutions (Lahaye et al. 1986). Such a fraction has also been isolated from other *Gracilaria*

Fig. 8. Degree of substitution of the agarobiose repeating unit with methoxyl group (DS Methyl) on carbon 6 of the galactose, and percent L-galactose-6-sulfate and L-galactose (L-gal) of the total 4-linked galactose (3,6-anhydrogalactose and L-galactose-6-sulfate + L-galactose) in agar extracted with cold-water (CX), boiling 80, 60, 40, 20% ethanol-water and water at 100 °C (0) and 121 °C (Au) from *Gracilaria crassissima*.

Table II. ¹H NMR chemical shifts assignments of agarose and 6-O-methylated agarose polymers from *Gracilaria crassissima*.

Proton ^a	Ref. Welti (1977) agarose	Sample <i>G. crassissima</i> agarose ^b	6-O-methylated agarose ^c
G1	4.56	4.55	4.55
G2	3.63	3.62	3.62
G3	3.76	(1)	(1)
G4	4.12	4.11	4.09
G5	3.71	(1)	(1)
G'6	3.80	(1)	(1)
G''6	3.75	(1)	(1)
Methyl			
A1	5.14	5.13	5.13
A2	4.10	4.11	4.10
A3	4.52	4.53	4.52
A4	4.66	4.66	4.65
A5	4.55	4.55	4.55
A'6	4.19	4.20	(1)
A''6	4.02	4.01	(1)

Chemical shifts in ppm relative to external DSS; (1) unresolved signals; ^a G and A refer to protons in the sugars depicted in Figure 1; ^b alkali-treated cold water extract; ^c 80% ethanol extract.

and *Gelidium* species (Lahaye *et al.* 1986, Lahaye 1986, Lahaye and Yaphe 1988, Lahaye and Yaphe, unpublished). Extraction of agaroses rich in precursor repeating units to agarobiose with cold water and 60% ethanol has been previously observed (Lahaye and Yaphe 1988, Lahaye *et al.* 1986).

¹³C NMR spectroscopic analysis of native and alkali-treated fractions from *G. tikvahiae* showed a variation in the concentration of 6-O-methylated agarobiose

Fig. 9. ¹³C NMR spectra of the native 80% ethanol extract (80), alkali-treated 60 (60AL), 40% (40AL) ethanol-water and water at 121 °C (AuAL) from *Gracilaria tikvahiae* (see Fig. 6 for legend); B and 4Me refers to carbon of the branch 4-O-methylated galactose residue and carbon of the methyl group from that sugar (see Fig. 1 for structure and Fig. 6 for conditions).

repeating unit in these extracts by the reduction in the intensity of the typical 6-O-methyl galactose signals (G', Fig. 9) and a concomitant increase of those of the galactose in the agarobiose repeat unit (G, Fig. 9). Previous reports mentioned 21 to 42% of 6-O-methyl-D-galactose in this agar (Craigie and Wen 1984, Duckworth *et al.* 1971). However, no signal for L-galactose-6-sulfate was observed on the spectrum of the native fractions. In contrast, a series of signals (B1: 98.5, B3: 70.7, B4: 79.4, B5: 72.7, B6: 61.4, 4-O-Me: 61.9 ppm, Fig. 10) were assigned to the proposed 4-O-methyl-L-galactose present as a branch in this agar (fig. 1, Craigie and Wen 1984). The resonance was assigned by comparing the chemical shifts observed for the signals on the spectrum of the 80% ethanol extract and those reported for the 4-linked α-L-galactose residue in an agar-type polymer obtained by solvolytic desulfation of α-L-galactose-6-sulfate rich agar (Lahaye *et al.* 1985), and by use of the DEPT NMR sequence (Fig. 19) for the assignment

of B6 and 4-*O*-methyl carbons. Indeed, as no signal for agarobiose repeating unit was observed on the spectrum of this fraction [no signal for G5 (75.3 ppm), G4 (68.7 ppm) were observed], the negative methylene signal at 61.4 ppm and the positive signal at 61.9 ppm were assigned to B6 and to the methyl carbon, respectively. Furthermore, the chemical shift of B1 at 98.5 ppm implies an α -type glycosidic linkage. However, the signal for B2 cannot be definitively assigned as it was expected to overlap with the resonance of A6 (69.4 ppm), A2 (69.9 ppm), or G'2 (70.2 ppm). Although most chemical shifts of the latter methylated sugar were assigned, it was not possible to determine at this time the residue of the main chain to which it was linked. The chemical shifts of carbons of the *D*-galactose proposed to be linked through G6 to C1 of the 4-*O*-methyl- α -*L*-galactose (Fig. 1, Hirase *et al.* 1983, Craigie and Wen 1984), were expected to be similar and thus, not resolved from those of the 6-*O*-methyl-galactose present in that agar. In addition, owing to rigidity of the trisubstituted branching residue (galactose or other) within the polymer, it is possible that the carbons of this residue can not fully relax during the repetition time of the spectral acquisition, thus yielding no or very broad signals that could not be distinguished from the noise on the spectrum. A similar situation was observed with the carbons of the branching glucose residue in amylopectin and glycogen (Dais and Perlin 1982). Although we have recently shown that oligomers produced from the hydrolysis of agars by β -agarase-I were excellent model compounds for the chemical structure analysis of agaroses (Rochas *et al.* 1986b, Lahaye 1986), we

were unable to produce small oligosaccharides substituted by 4-*O*-methyl- α -*L*-galactose probably because of the high concentration of 6-*O*-methylated agarobiose repeating unit in this agar. The agarase used in the present study is known to have a very low activity on such methylated disaccharide repeating unit (Lahaye 1986, Morrice *et al.* 1983).

The presence of 4-*O*-methyl-*L*-galactose in agar has been observed in commercial and *Gelidium amansii* Lamouroux agar (Araki *et al.* 1967), *Laurencia undulata* Yamada (Hirase *et al.* 1983), *Gracilaria tikvahiae* McLachlan, *G. cervicornis* (Turner) J. Agardh (*ferox*), *G. damaecornis* J. Agardh, *G. domingensis* Sonder ex Kützinger pro. Syn., *G. verrucosa* (Hudson) Papenfuss (Bird *et al.* 1987, Craigie and Wen 1984, Duckworth *et al.* 1971, Lahaye 1986), and *G. salicornia* (C. Agardh) Daws (Ji *et al.* unpublished) and further work is required to determine its effects on the physical and rheological properties of agar.

Gel strength

The gel strength of alkali-treated *G. crassissima* agar fractions are reported in Table I. Values obtained for the cold-water and 60% ethanol extracts were similar to that of a commercial bacteriological grade agar (Difco-Bacto, 214 g/cm²), whereas the gel strength of the 40% ethanol extract was greater than that obtained for a commercial agarose sample (Seakem, lot # 711801, Marine Colloids FMC Corp., 3423.7 N/cm², Table I). These results confirmed a previous report (Lahaye and Yaphe 1988) showing that different gel strengths were obtained between fractions extracted from the same alga. As the chemical characteristics of these fractions determined by ¹³C, ¹H NMR and IR spectroscopy were close, differences in molecular weight of these agars may be responsible for such variations in gel strength. Sulfated polysaccharides can be degraded to a low extent by dialysis (Goring 1954). The amount of degradation that occurred during the agaroses preparation due to dialysis may be sufficient to promote the observed gel strength differences. Further work is currently being conducted to determine the molecular weight of these fractions. Nevertheless, the gel strengths and the chemical characteristics of the alkali-treated fractions from *G. crassissima* are indicative of a high quality agarose suitable for industrial and biotechnological applications.

Gel strength was not measured for *G. tikvahiae* agar extracts, but the fraction of the alkali-treated autoclaved agar eluted from DEAE-Sephadex A 50 with distilled water failed to gel at 5% concentration. The

Fig. 10. DEPT NMR spectrum of the native 80% ethanol extract from *Gracilaria tikvahiae* (2037 transients, see Figs 6 and 8 for legend).

presence of 4-*O*-methyl- α -*L*-galactose on the molecules may partly be responsible for the lack of gelling ability or this fraction may represent degraded molecules arising from long storage of the seaweed (Young and Goring 1958), autoclave extraction, or the dialysis step in the preparation of the fractions.

X-Ray diffraction of the algal residues

Chemical treatment of the residues recovered after agar extraction from *G. crassissima* and *G. tikvahiae* yielded 3.8 and 4.0%, respectively, of insoluble fibers on the basis of the starting algal dry weight. These values are in agreement with those reported for other marine red algae (Gretz *et al.* 1986, Young 1966). The X-ray diffraction pattern of these fibers from *G. crassissima* was that of cellulose-I and, after mercerization with 10% NaOH, that of cellulose-II (Table III). Similar diffraction patterns and spacings were obtained from *G. tikvahiae* fibers (data not shown). It is known that X-ray spacings of native cellulose vary appreciably from one species to another, and in particular, the spacing at 0.399 nm in Table III can vary from 0.392 to 0.403 nm (Okano and Koyanagi 1986). When this value is higher than average, as in the present work, it is generally attributed to the low crystallinity of the cellulose (Revol *et al.* 1987). This is confirmed by the full mercerization of the cellulose sample obtained at an aqueous sodium hydroxide concentration as low as 10%. Usually, concentrations of 14% or higher are required to obtain a full conversion from cellulose I to cellulose II crystal form (Warwicker *et al.* 1966).

Cellulose is known to compose part of the fibrils in the cell-walls of the conchocelis phase of marine red algae from the Bangiophyceae (Gretz *et al.* 1980, Mukai *et al.* 1981). The present results definitively establish the presence of cellulose in algae belonging to the other class of the Rhodophyta, the Florideophyceae, as up to now doubts existed (McCandless 1978). However, the occurrence of cellulose in all the generic phases of these algae remains to be shown.

In conclusion, the present results demonstrate that agar synthesized by *G. crassissima* is of excellent chemical qualities with an overall low methoxyl content and a high agarobiose concentration once the biological precursor repeating units have been modified by an alkali treatment. Thus, this alga represents an excellent potential commercial source of agarose.

The different extractions patterns obtained from *G. crassissima* and *G. tikvahiae* most probably reflected

Table III. Interplanar spacings (nm) of cellulose I (A, native cellulose) and cellulose II (B, mercerized cellulose) from *Gracilaria crassissima*.

A	B	Ref. ¹
0.610	0.730	0.729
0.543	0.442	0.445
0.439	0.407	0.402
0.399	0.310	0.317
0.319	0.258	0.259
0.258		

¹ Ref. refers to values published by Kolpak *et al.* 1978.

the presence of cross-linkages between cell-wall polymers in the latter alga. Further research on the organisation of the algal cell-wall polymers should give information for selecting enzymes that break these cross-linkages and to design extraction procedures or both, that would increase the yield and quality of agar.

Anion-exchange chromatography of agar fractions obtained by the sequential solvent method of these two species of algae demonstrated that they are not chemically homogeneous, and thus, combination of both sequential extraction and anion-exchange chromatography of agar represents an excellent means for obtaining purified agaroses that should prove useful in studies relating chemical structure to physical and rheological properties of these polymers.

The assignment of ¹³C NMR signals associated with agarose containing 4-*O*-methyl- α -*L*-galactose will be of diagnostic value for the detection of this structure in agar from other species. However, further work is required to determine the exact linkage of this methylated sugar on agarose and the effect of this sugar on the rheological and physical properties of agarose.

Finally, X-ray diffractometry definitively demonstrated the presence of cellulose as part of the fibrillar material in the cell-walls of Gracilariaceae. However, a thorough investigation of the chemical nature of all the fibrillar material in these algal cell walls is needed.

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