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Effects of Seasons on the Chemical Structure and Gel Strength of *Gracilaria pseudoverrucosa* Agar (Gracilariaceae, Rhodophyta)*

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ABSTRACT

The seasonal effects on the chemical structure and rheological properties of Gracilaria pseudoverrucosa *agar have been investigated using a sequential solvent extraction, ^{13}C NMR and infrared spectroscopy, and gel strength measurements. The results showed that agar enriched in precursor to the agarobiose repeat unit were obtained from algae collected in summer. In contrast, algae collected in winter contained agar molecules richer in alkali-stable sulfate groups attributed in part to* D*-galactose-4-sulfate. A similar total concentration of 6-O-methylated agarobiose repeat units was present in the agar from both algal samples but the distribution of the methylated disaccharide varied in the fractions. Agar fractions from the summer-collected sample had higher gel strength than those of the winter ones. Alkali treatment markedly improved the gel strength of the agar from the summer harvested seaweed. Different gel strengths were observed for the native and alkali-treated agar fractions extracted from the same algal sample and a gel strength comparable to that obtained for a commercial bacteriological grade agar was obtained from the alkali-treated 40% ethanol extract agar from the summer collected alga. The chemical and rheological variations due to seasonal changes are interpreted as reflecting the ratio of actively-growing (young) to resting (old) tissue in the alga and are proposed to represent a type of 'secondarization' of the algal cell-wall.*

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INTRODUCTION

The agar polysaccharides are the matrix component of the cell walls of certain marine red algae referred to as agarophytes (Tseng, 1945). These water-soluble polysaccharides are composed of basic repeat units consisting of β (1→4) linked D-galactopyranose alternating with α (1→3) linked 3,6-anhydro-L-galactopyranose, named agarobiose (Araki, 1966; Fig. 1(a)). This disaccharide is often substituted by methoxyl, sulfate esters and/or pyruvate ketal groups and can also occur under its biological precursor form where L-galactose-6-sulfate replaces the 3,6-anhydro-L-galactose residue (Fig. 1(b), Rees 1961*a*; Araki, 1966; Duckworth & Yaphe, 1971; Duckworth *et al.*, 1971; Lahaye *et al.*, 1986). Agar and one of its components, agarose, which is virtually free of substituent group, form gels or viscous solutions at low concentrations in water and have, for these reasons, many biochemical, biological and industrial applications (Meer, 1980). The gelling mechanism and the physical properties of these molecules are closely related to their chemical structure (Rees, 1969).

Mariculture of *Gracilaria* species is being investigated for the commercial production of agar (Friedlander & Lipkin, 1982; Craigie *et al.*, 1984; Hanisak & Ryther, 1984) but this project is facing the problem of variability of the chemical and rheological properties of agar. These variations were demonstrated between species and strains (Duckworth *et al.*, 1971; Patwary & van der Meer, 1983; Craigie *et al.*, 1984; Ji *et al.*, 1985; Lahaye *et al.*, 1986), and according to environmental factors (Bird *et al.*, 1981; Craigie & Wen, 1984; Craigie *et al.*, 1984; Christiaen *et al.*, 1987). Numerous reports have shown the effects of seasonal variations on the property of *Gracilaria* agar (John & Asare, 1975; Oza, 1978; Kim & Henriquez, 1978; Hoyle, 1978; Asare, 1980; Whyte *et al.*, 1981; Yang *et al.*, 1981; Nelson *et al.*, 1983) but contradictions in the results exist (Duraitnam & Santos, 1981; Doty *et al.*, 1983). In fact, it is difficult to compare these data partly because different methods were used to prepare the agars and, probably, because the nature of seasonal changes varies geographically such as, for example, between the tropics and temperate areas. Under the latter climate, the seasonal changes are usually associated with, at least, marked variations in water temperature, light intensity and photoperiod. In particular, these factors were shown in laboratories to affect growth and agar biosynthesis in *Gracilaria* species (Craigie & Wen 1984; Christiaen *et al.*, 1987).

We now report on the modifications of the chemical structures and changes in the gel strength of agar synthesized by *Gracilaria pseudoverrucosa* grown in nature in different seasons. Accounts on the taxonomy

of this alga and on the basic chemical constituents of its agar have previously been reported by Whyte & Englar (1980). Samples of seaweed were collected in summer and winter, and families of agar polysaccharides were extracted by the sequential solvent method described by Lahaye *et al.* (1986). Gel strengths of native and alkali-treated agar fractions were measured, and chemical characteristics were determined using ^{13}C NMR and infrared spectroscopy.

MATERIALS AND METHODS

G. pseudoverrucosa samples collected in September 1984 and January 1985 in Wiseman's Bay, British Columbia, Canada were a gift from W. N. Wheeler at the Bamfield Marine Station (Bamfield, B. C., Canada). The air-dried shipped and stored algae were hand sorted, broken into small pieces (maximum length of 2 mm), freeze-dried and extracted according to the method of Lahaye *et al.* (1986) with the exception of the boiling 20% ethanol-water and autoclaved extracts which were omitted. All extracts were dialyzed extensively against distilled water, concentrated *in vacuo*, filtered hot with pressure (0·45 μm pore size membranes, Millipore Corp., Canada), and freeze-dried. The yield of each agar fraction reported in the text refers to a percentage of the total dry weight of polymer extracted from the dry seaweed samples.

All the agar fractions except that of the 80% ethanol extracts were alkali-treated according to a modification of Rees' method (1961*b*) as described (Lahaye *et al.* 1986).

Proton decoupled ^{13}C NMR spectra of 4–5% (w/v) polysaccharide solutions in D_2O at 80°C were recorded on a Bruker WH 400 spectrometer (Bruker, Canada) at 100·6 MHz with spectral windows of 10–18 KHz and relaxation delays of 0·5 to 0·7 s. Chemical shifts were measured in part per million (ppm) from internal dimethyl sulfoxide (DMSO) and converted to values related to tetramethylsilane (TMS, conversion constant: 39·6).

Infared spectra of agar films were recorded on a Perkin-Elmer 297 Infrared Spectrophotometer (Perkin-Elmer Corp., Canada). Agar films were prepared and ratio of absorbances for semi-quantitative comparison of 3,6-anhydrogalactose and total sulfate concentrations were done according to the method of Rochas *et al.* (1986). The very low concentration of galactose-4-sulfate in the agar fractions that contributes to the absorbance at 930 cm^{-1} (Rochas *et al.* 1983) was neglected when estimating the variation of 3,6-anhydrogalactose contents in agar fractions.

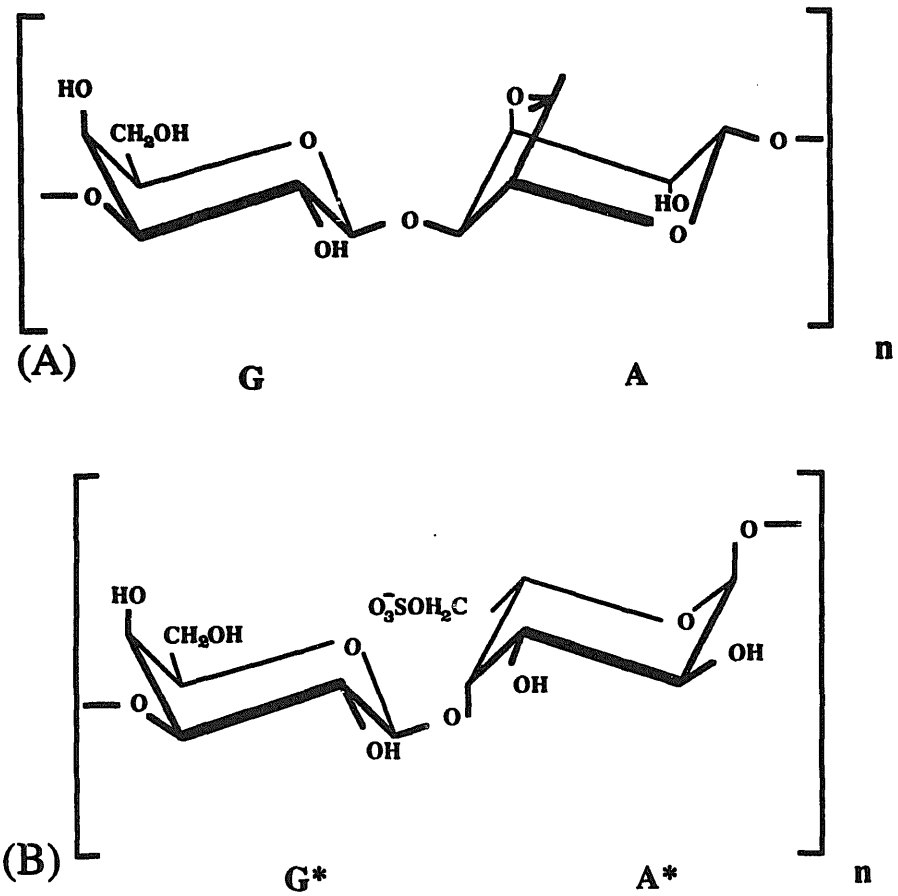

Fig. 1. Structure of the agarobiose repeat unit (A) and its biological precursor (B). (A) 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose; (B) 4-*O*-β-D-galactopyranosyl-L-galactose-6-sulfate. G, G*: D-galactose; A: 3,6-anhydro-L-galactose; A*: L-galactose-6-sulfate.

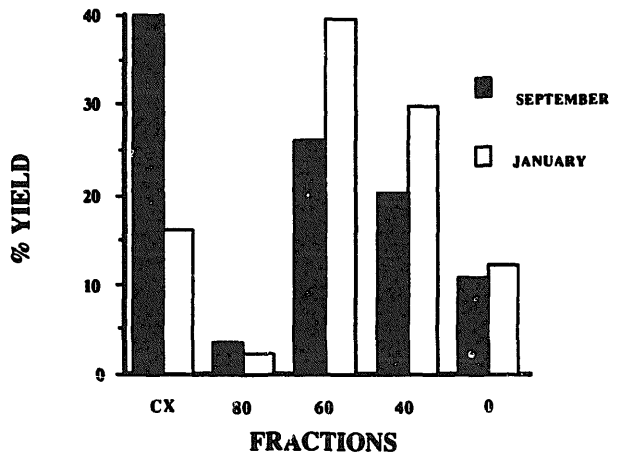

Fig. 2. Yields of agar from *Gracilaria pseudoverrucosa* collected in September and January extracted with water at 22°C (CX), boiling 80, 60, 40% ethanol-water, and water at 100°C (80, 60, 40, 0, respectively). The yield in each extract represents a weight percentage of the total polymer extracted from the alga.

The gel strength of various agar fractions was measured using the semimicro gelometer as described by Goring (1956). Agar solutions (1% w/v, 4 ml) were allowed to gel at room temperature in plastic vials (2·2 cm diameter, 2 cm height) covered with Parafilm and left overnight at 4°C. The gel strength measurements were done in triplicate on gels that had been warmed to room temperature for 30–60 min.

RESULTS

Extraction of *G. pseudoverrucosa* agar

The total yield of agar polymers extracted from dry weight of *G. pseudoverrucosa* collected in September and January was 35·9 and 38·0%, respectively. The yields of agar from the two algal samples soluble in water at 22°C (CX), boiling 80%, 60%, 40% ethanol, and water at 100°C (0) are illustrated in Fig. 2. The cold water extract (CX) was a major fraction (39·9%) of the agar from algae collected in September as compared with that of the agar from algae harvested in January (CX, 16·0%). Major fractions of agar were extracted from both samples of boiling 60% and 40% ethanol with small differences in yield.

^{13}C NMR spectroscopy

The ^{13}C NMR spectra of all the fractions (native and alkali-treated) except that of the 80% ethanol extracts, showed 12 major signals attributed to agarobiose repeat units (Usov *et al.*, 1980; Fig. 3). The spectra of the native 80% ethanol extracts had major signals attributed to 6-*O*-methylated agarobiose repeat units (Nicolaisen *et al.*, 1980; Fig. 3). Minor signals for 6-*O*-methylated agarobiose repeat units were observed on the ^{13}C NMR spectra of native and alkali-treated 60% ethanol extracts, alkali-treated cold water, 40% ethanol, and hot water extracts.

The methoxyl content estimated from the integral of the signal for G6 (61·4 ppm, C-6 of β-D-galactopyranose in agarobiose) or G*6 on the spectrum of native 80% ethanol extract (61.5 ppm, C-6 of β-D-galactopyranose in the agarobiose biological precursor) and G'6 (71·8 ppm, C-6 of 6-*O*-methyl-β-D-galactopyranose in 6-*O*-methylated agarobiose), was higher in alkali-treated cold water (18%) and 40% ethanol (13%) fractions from algae collected in January than in the alkali-treated cold water (7%) and 40% ethanol (7%) fractions from algae harvested in September (Table 1). However, this low concentration of methylated

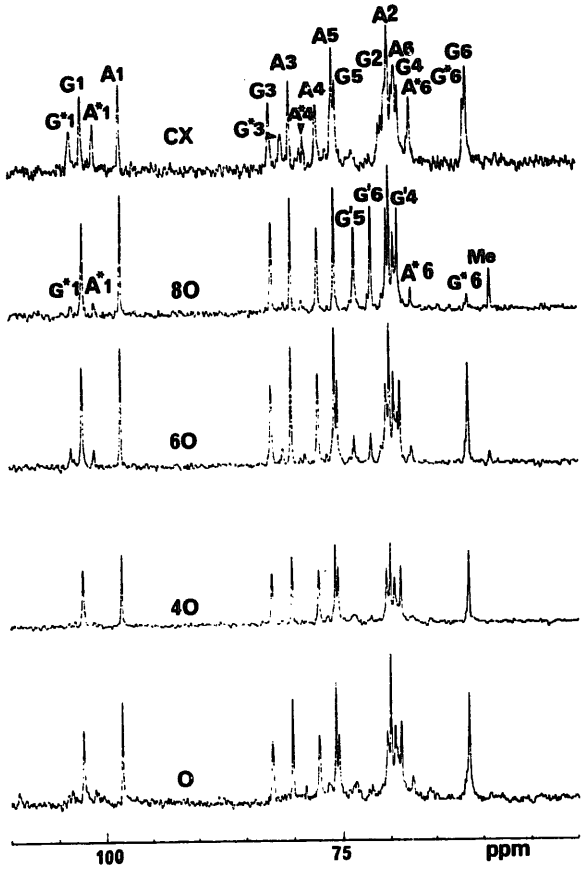

Fig. 3. ^{13}C NMR spectra of *Gracilaria pseudoverrucosa* agar extracted from the September sample with cold water (CX), boiling 80, 60, 40% ethanol–water and water 100°C (80, 60, 40, 0, respectively). A* and G* refer to carbons in the precursor repeat unit to agarobiose; A*′ and G*′ refer to carbons in the 6-*O*-methylated precursor repeat unit to agarobiose (6-*O*-methyl-4-*O*-*β*-D-galactopyranosyl-L-galactose-6-sulfate); A and G refer to carbons in the agarobiose repeat unit; G′ and CH_3 refer to carbons in the 6-*O*-methyl-*β*-D-galactose of the 6-*O*-methylated agarobiose repeat unit (6-*O*-methyl, 4-*O*-*β*-D-galactopyranosyl-3,6-anhydro-L-galactose).

agarobiose repeat units in the alkali-treated cold water extract from algae collected in September resulted from a dilution of these repeat units by unsubstituted agarobiose repeat units. When yields are taken into account, an identical concentration of methylated repeat units (2%) was extracted by water at 22°C from the January and September algal samples (Table 1). In fact, although a shift in the methoxyl content distribution was observed between the agar fractions from the two algal samples, the total per cent concentration of 6-*O*-methylated agarobiose repeat units was similar (16%) in the agars from algae collected in September and January (Table 1).

TABLE 1
Concentration of 6-*O*-Methylated Agarobiose Repeat Units in Agar Fractions Extracted from *Gracilaria pseudoverrucosa* Collected in September and January

Fraction	*September*			*January*		
	A	*B*	*C*	*A*	*B*	*C*
CX	28·3	7	2	10·5	18	2
80	3·5[a]	88	3[a]	2·4[a]	100·0[b]	2[a]
60	22·1	33	7	33·1	21	7
40	16·2	7	1	23·1	13	3
0	7·0	27	2	8·4	25	2
Total	76·5[c]		16[d]	76·9[c]		16[d]

A, weight percentage of agar polymers recovered after alkali treatment from the total weight of polymer extracted from the alga; *B*, percent concentration of 6-*O*-methylated agarobiose repeat units in alkali-treated agar fractions calculated from the integral of ^{13}C NMR signal for C-6 of β-D-galactopyranose (G6) and C-6 of 6-*O*-methyl-β-D-galactopyranose (G'6); and *C*, percent concentration of total 6-*O*-methylated agarobiose repeat units extracted from the algal samples ($A \times B/100$), recovered in alkali-treated cold water (CX), native 80% (80), alkali-treated 60% (60), 40% (40) ethanol, and hot water (0) extracts.

[a]Value for native polymers.

[b]Overestimated value as the integral of G6 was not measured.

[c]Does not include the 80% ethanol extract.

[d]Includes the 80% ethanol extract.

Signals attributed to disaccharide sequences containing L-galactose-6-sulfate were detected in the spectra of native cold water and 60% ethanol extracts (Fig. 3. A*, G*; Lahaye *et al.*, 1985). Signals for L-galactose-6-sulfate residues barely exceeded the noise level in the spectrum of the 80% and 0% ethanol extracts (Fig. 3) and did not permit a clear observation of sequence effects on the chemical shifts of methylated agarobiose and precursor repeat units on the spectrum of the 80% ethanol extracts as described by Morrice *et al.* (1983). The concentration of L-galactose-6-sulfate in polymers extracted with water at 22°C from algae collected in September was higher (46%) than that from algae harvested in January (29%) as estimated from the integral of the signal for A*1 (101·3 ppm, C-1 of the α-L-galactopyranose-6-sulfate residue) and A1 (98·3 ppm, C-1 of the 3,6-anhydro-α-L-galactopyranose residue) on the ^{13}C NMR spectra.

Small signals attributed to repeat units containing D-galactose-4-sulfate (A°1: 96·8, G°4: 77·0, G°5: 75·0, and G°2: 70·8 ppm, Fig. 4;

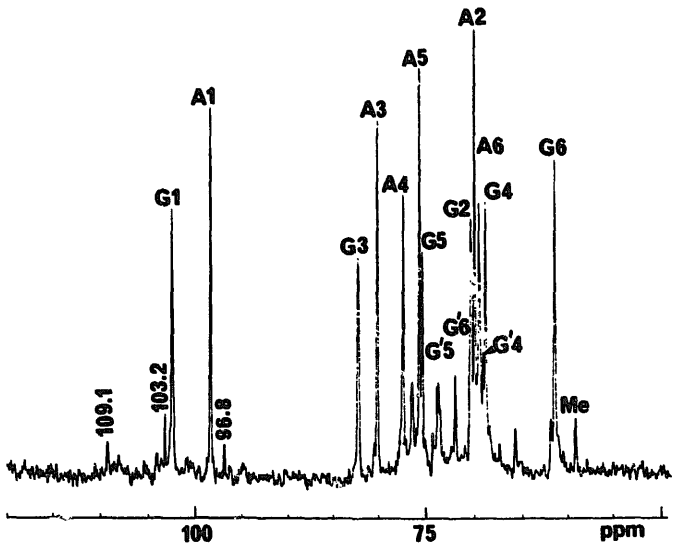

Fig. 4. ^{13}C NMR spectrum of the alkali-treated hot water extract agar (fraction 0) from *Gracilaria pseudoverrucosa* collected in January. A and G refer to carbons in the agarobiose repeat unit.

Lahaye, 1986; Lahaye *et al.*, in preparation) were observed in all the ^{13}C NMR spectra of alkali-treated fractions from *G. pseudoverrucosa* collected in September and January. A series of unidentified ^{13}C NMR signals at 109·1, 67·4, 65·6, and 61·8 ppm was observed in the spectra of alkali-treated agar fractions extracted with hot water (100°C; Fig. 4) and, in part, in the spectrum of the alkali-treated cold water extract from algae collected in January. Other small signals at 103·2, 76·5, 76·4, and 73·8 ppm on the ^{13}C NMR spectra of alkali-treated agar fractions from both algal samples also remain unattributed (Fig. 4). The intensity of this last series of signals increased on the spectra from 60% ethanol to hot water extracts, and was higher in the spectra of alkali-treated agar fractions from algae harvested in January that those from algae collected in September. These series of peaks may correspond to carbons of other cell wall polysaccharides and their attribution is under investigation. No ^{13}C NMR signal was observed for floridean starch.

Infrared spectroscopy

Infared spectra of native and alkali-treated agar polymers recovered from the cold water, 60%, 40% ethanol, and hot water extracts from both algal samples, exhibited absorbances typical of agar (Christiaen & Bodard, 1983; Rochas *et al.*, 1986; Fig. 5): between 2890 and 2940

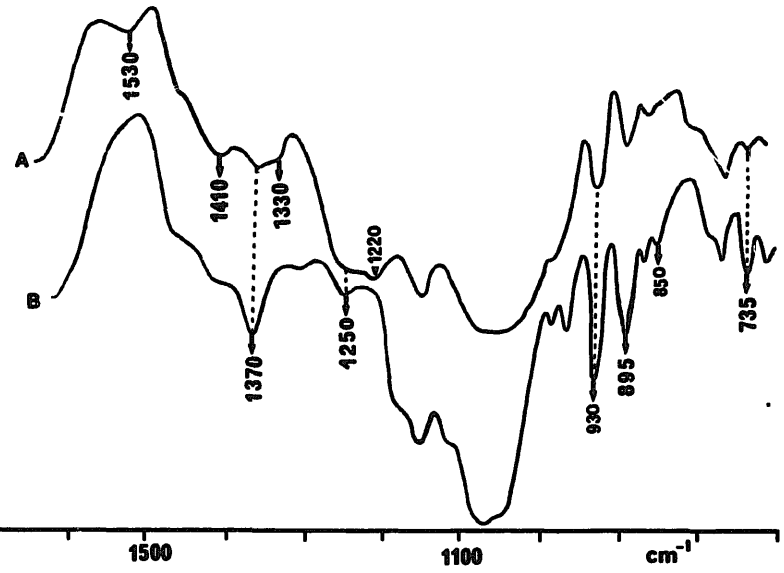

Fig. 5. Infrared spectrum of the cold water extract agar from *Gracilaria pseudoverrucosa* collected in September: (A) before and (B) after alkali treatment.

cm^{-1} for CH_2 and CH groups (Colthup, 1950); at 1410, 1370, 1335, 1250 and 1220 cm^{-1} for sulfate esters (Detoni & Hadzi, 1957; Lloyd *et al.* 1961; Akahane & Izumi, 1976; Rochas *et al.*, 1986); and at 930 cm^{-1} for 3,6-anhydrogalactose (Stanley, 1963).

Native polymers had a band at 1530 cm^{-1} attributed to N—H linkages in proteins (Bellamy, 1975; Fig. 5, A). Native cold water and 60% ethanol extracts had a shoulder at 820 cm^{-1} assigned to galactose-6-sulfate (Stancioff and Stanley, 1969; Fig. 5, A) The IR spectra of alkali-treated polymers had no absorbance at 1530 or 820 cm^{-1}, and showed a shoulder at 850 cm^{-1} attributed to galactose-4-sulfate (Lloyd *et al.*, 1961; Fig. 5, B).

The values obtained for 3,6-anhydrogalactose (Fig. 6) and total sulfate (Fig. 7) contents of native and alkali-treated agar fractions extracted from algae collected in January and September, were divided by their respective ratios of absorbances obtained from commercial agarose (Seakem, Marine Colloids F.M. Corp. lot no. 711801). A value of 1·0 in Figs 6 and 7 represents the concentration of 3,6-anhydrogalactose and sulfate in the sample of commercial agarose. High sulfate and low 3,6-anhydrogalactose contents were observed in native polymers extracted with water at 22°C. The content of sulfate decreased in the 60% and 40% ethanol extracts and increased in the hot water extracts (0) (Fig. 7). The concentration of 3,6-anhydrogalactose followed a reverse pattern (Fig. 6). These results are in agreement with previous observations (Lahaye *et al.*, 1986).

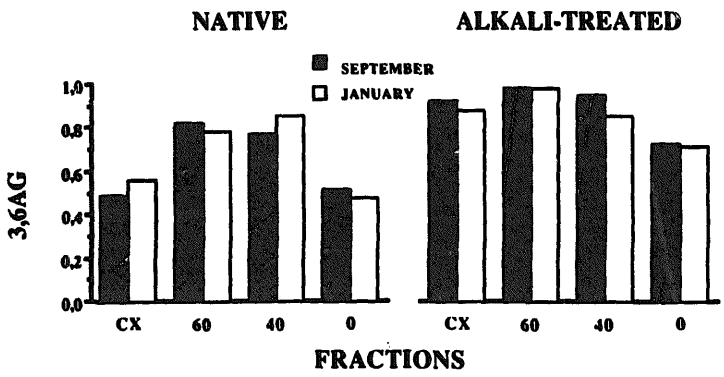

Fig. 6. Relative concentration of 3,6-anhydrogalactose in native and alkali-treated agar fractions extracted from *Gracilaria pseudoverrucosa* collected in September and January. The relative concentrations are expressed in arbitrary units (3,6-AG) calculated from the ratio of IR absorbances at 930 cm^{-1} (3,6-anhydrogalactose) to 2920 cm^{-1} (CH, total sugar) in the extracts and in a commercial agarose sample used as reference. (930/2920 sample / 930/2920 agarose = 3,6AG)

Alkali treatment dramatically affected sulfate and 3,6-anhydrogalactose contents (Figs 7 and 6). The alkali-treated agar fractions from algae collected in September (except the hot water extract) had sulfate and 3,6-anhydrogalactose contents similar to that of commercial agarose, indicating that most of the sulfate was associated with L-galactose-6-sulfate in the native polymers. Alkali-treated extracts from the January sample had a high anhydrogalactose content (Fig. 6) but, in contrast to the alkali-treated extracts from the September alga, the sulfate concentration was high compared with commercial agarose (Fig. 7). This suggested that only part of the sulfate groups in the native cold water extract agar were associated with L-galactose-6-sulfate. The remaining sulfate was atributed to alkali-stable sulfate groups on sugar residues in this polysaccharide fraction.

A marked increase in absorbance at 1370 and 735 cm^{-1} was observed on the IR spectra of alkali-treated fractions and, particularly, that of the cold water extracts (Fig. 5). For example, on the spectrum of the cold water extract from September algae, the ratio of absorbances at 1370/2920 cm^{-1} and 735/2920 cm^{-1} increased from 0·308 to 0·602 and from 0·118 to 0·463 respectively.

The low 3,6-anhydrogalactose (930/2920 cm^{-1}) content of alkali-treated hot water extracts from both algal samples may indicate the presence in these fractions of non-agar polymers that contributed to the total sugar content (2920 cm^{-1}). These fractions also had a high sulfate content (1250/2920 cm^{-1}; Fig. 7) before and after alkali treatment, indi-

cating substitution of the sugar units with alkali-stable sulfate groups. These results agree with previous observations (Lahaye *et al.*, 1986).

No major change was observed in the 3,6-anhydrogalactose content between the native and alkali-treated 40% ethanol extract from January algae, indicating a possible degradation of the anhydride on alkali treatment.

Gel strength

Gel strengths of native and alkali-treated cold water, 60%, 40% ethanol extracts, and alkali-treated hot water extracts are shown in Fig. 8. Native polymers had no, or low, gel strength with relatively higher gel strength for algae collected in September. Alkali treatment markedly improved the gel strength of agar fractions from algae collected in September but

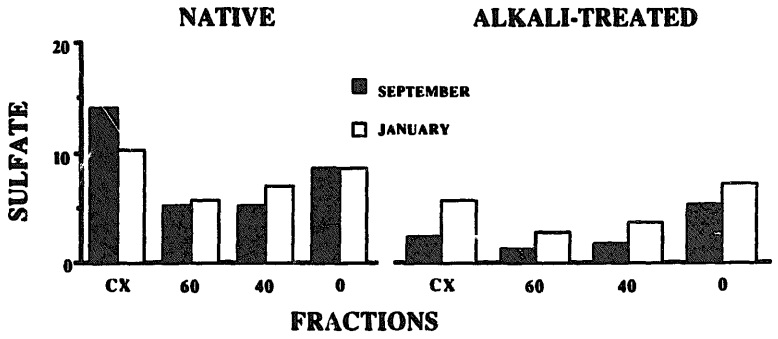

Fig. 7. Relative concentration of sulfate in native and alkali-treated agar fractions extracted from *Gracilaria pseudoverrucosa* collected in September and January. The relative concentrations are expressed in arbitrary units calculated from the ratio of IR absorbances at 1250 cm^{-1} (total sulfate) to 2920 cm^{-1} (CH, total sugar) in the extracts and in a commercial agarose sample used as reference. (1250/2920 sample / 1250/2920 agarose = sulfate)

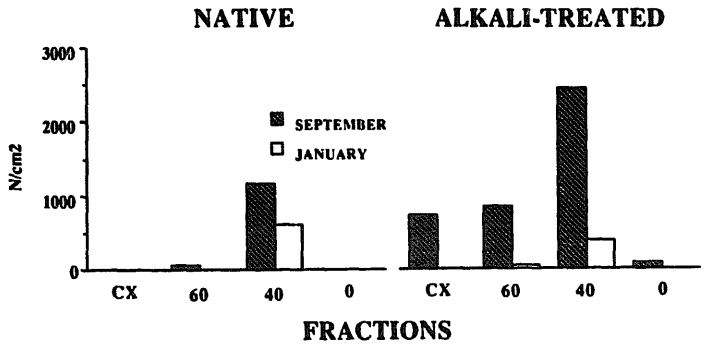

Fig. 8. Gel strength of 1% (w/v) solution of native and alkali-treated agar fractions extracted from *Gracilaria pseudoverrucosa* collected in September and January.

had little effect on those of agar fractions from algae collected in January. A higher gel strength was obtained for the 40% ethanol extracts than for the other fractions. The gel strength of alkali-treated 40% ethanol extract from algae collected in September (2452.5 N/cm^2) was comparable to that of a commercial agar (2099.3 N/cm^2, Difco-Bacto).

DISCUSSION

Major differences were observed in the yields, structure, and quality of agar fractions extracted from the summer and winter samples of *G. pseudoverrucosa*. In agreement with previous observations (Lahaye *et al.*, 1986), the sequential solvent extraction method allowed the isolation of agars enriched with L-galactose-6-sulfate, 6-*O*-methylated agarobiose repeat units and also disaccharides repeat units substituted by alkali-stable sulfate groups. It also prevented the loss of polysaccharides associated with the freeze–thaw and alcohol precipitation procedures (Wen & Craigie, 1984; Lahaye, 1986).

The samples of algae collected in September and January represented plants under two different growth statuses: active growth and resting conditions, respectively. These samples were expected to have different ratios of young to mature tissue and thus, different types of agar. Indeed, Craigie & Wen (1984) showed that young tissue from actively-growing *G. tikvahiae* contains agar enriched with L-galactose-6-sulfate residues, a precursor to 3,6-anhydrogalactose (Rees, 1961*a*) whereas older algal tissues were rich in agar substituted by methoxyl and alkali-stable sulfate groups.

As expected, actively growing *G. pseudoverrucosa* collected in September had a high concentration of cold water (22°C) soluble polymers (39·9%; Fig. 2, CX) enriched with repeat units containing L-galactose-6-sulfate (46%, Fig. 3). These polymers were attributed to newly synthesized agar associated with young algal tissues. In contrast, algae harvested in January yielded a lower concentration of cold water soluble agar polymers (16·0%, Fig. 2, CX) containing fewer repeat units with L-galactose-6-sulfate (29%). The fractions of agar from this algal sample also contained higher concentrations of alkali-stable groups (Fig. 7) associated in part with D-galactose-4-sulfate (Fig. 4), and thus, was taken as characteristic of polysaccharides associated with older algal tissues.

Similar variations in the yield of cold water extract agar in relation with time of algal collection have also been reported for *G. tikvahiae* mutants (Cote & Hanisak, 1986).

However, the increase in methoxyl content expected in agar fractions from non-growing algae was not observed. Instead, a shift was observed in the distribution of methylated repeat units from the 80% and non-gelling 60% ethanol soluble agar polymers of actively-growing algae, to the gelling 40% ethanol soluble agar fraction of algae collected in January (Table 1). The depigmentation procedure, involving boiling 80% ethanol washes, and the freeze-thaw cycles used in the preparation of *G. tikvahiae* agar (Craigie & Wen, 1984) may have removed fractions of sulfated and/or methylated agar from young actively-growing tissues. These losses would explain the apparent increase in yield and methoxyl content of agar extracted from mature *G. tikvahiae* tissues.

The structural and/or physiological functions in the cell wall and cells in the algae that particularly synthesize the low concentration of highly methylated agar extracted with boiling 80% ethanol-water (see also Lahaye, 1986; Lahaye *et al.*, 1986) remain to be established.

The gel strength of agar fractions was also affected by the growing status of the algae and thus, reflected the degree of chemical structure modification of the 'young' and 'mature' polysaccharides. The differences observed between the gel strength of alkali-treated agar fractions extracted from *G. pseudoverrucosa* collected in September and January (Fig. 8) are in agreement with the results of Craigie & Wen (1984). They showed that alkali-modified agar polymers extracted from young growing tissue of *G. tikvahiae* have higher gel strengths than those extracted from old tissues. The lower gel strength of agar fractions extracted from algae collected in January (Fig. 8) was probably related to the presence of D-galactose-4-sulfate and to the unidentified structures observed by ^{13}C NMR spectroscopy (Fig. 4).

In contrast, Cote & Hanisak (1986) observed higher gel strengths for the hot water extract agar from *G. tikvahiae* mutants collected in winter as compared with those extracted from algae collected in spring. The different results may be explained by the fact that these extracts were not alkali-treated and that, based on the sulfate and 3,6-anhydrogalactose contents reported by the authors. the spring algal extracts appeared to contain higher concentrations of precursor repeat units.

Differences in gel strengths between fractions of agar extracted from the same algal sample (Fig. 8) have previously been observed for *G. crassissina* (Lahaye, 1986), and await further work in order to be explained. Molecular weight differences may account for such variations (Mitchell, 1980).

The methoxyl content and gel strength of agar extracted from *G. pseudoverrucosa* collected in September agree with the previously published data of Whyte & Englar (1980). The low yield of agar (11·3%)

reported by these authors may be the result of extensive washings of the dry alga with distilled water prior to the hot water extraction. These washings may have removed the cold water soluble agar fraction.

Infrared absorbances can be used to determine the sulfate content of agars and carrageenans (Rochas *et al.*, 1986). The increase of absorbance at 1370 and 735 cm^{-1} observed in this work after the alkali conversion of L-galactose-6-sulfate to 3,6-anhydro-L-galactose (Fig. 5) may be related to the presence of the anhydride sugar in the polymers. Recently, Malfait *et al.*, (1987) suggested the attribution of the Raman band at 730–740 cm^{-1} to a complex ring-vibration mode of the carbohydrate backbone in carrageenan (a closely related 3,6-anhydrogalactose containing sulfated red algal galactan). Further work should determine if these absorbances at 735 and 1370 cm^{-1} can be used for the quantitative determination of 3,6-anhydrogalactose in agar and/or carrageenans. In any case, caution is required when using absorbance at 1370 cm^{-1} for the determination of sulfate contents in agars and carrageenans (Rochas *et al.*, 1986) as it may be affected by the concentration of 3,6-anhydrogalactose in the polysaccharide.

In conclusion, these results clearly confirm the seasonality of agar structure and quality. This most likely reflects the ratio of actively-growing to mature tissue in *G. pseudoverrucosa* and probably occurs in other *Gracilaria* spp. as well. Actively-growing algae synthesize high concentrations of 'young' agar enriched with L-galactose-6-sulfate forming strong gels after alkali modification. Substitution of agar with D-galactose-4-sulfate (and other, unidentified, structures) was associated with the maturation of *G. pseudoverrucosa* tissue. The chemical modifications of polysaccharides due to aging may differ between species or strains of algae and may be seen as a sort of 'secondarization' of the algal cell wall by analogy to higher plants cell-walls. The matricial polysaccharides in the 'primary' wall would consist of agar enriched in precursor repeat unit with physical properties that would decrease mechanical constraints for the elongating and dividing cells of the actively growing tissues. Such molecules do not gel (cold water extracts) and divalent cations (Ca^{2+} and/or Mg^{2+}, for example) would help in stabilizing them in the cell-wall. As the algal tissues age, fewer cell divisions occur and the thicker cell walls have to act as a flexible but tough skeleton. Substitution of the agar polymers with chemical groups and/or the formation of cross-links would increase the cohesiveness and rigidity of the cell wall in addition to playing other biological functions such as providing a means of resisting pathogens.

It is now clear that the chemical heterogeneity of agar synthesized by *Gracilaria* species, and maybe of other familiies, is more complex than

was previously described (Duckworth & Yaphe, 1971; Duckworth *et al.*, 1971; Lahaye *et al.*, 1986). The concentration and distribution of substituent groups on agar, and hence, the solubility and physical properties of the polymers, vary according to the physiological conditions under which the alga is grown. Prior knowledge of the type of variations existing within *Gracilaria* and other species is especially important when harvesting the algae for commercial production of agar.

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