Influence of nitrogen availability on agar-polysaccharides from *Gracilaria verrucosa* strain G-16: structural analysis by NMR spectroscopy

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Abstract

The influence of nitrogen availability on the chemical structure of agar polymers isolated from *Gracilaria verrucosa* strain G-16 was ascertained by sequential solvent extraction and carbon 13 nuclear magnetic resonance (^{13}C NMR) spectroscopy. Agar isolated from *G. verrucosa* strain G-16 cultured under nitrogen limited conditions showed large non-polar components but produced spectra indicative of only minor amounts of methylation. These agars also produced spectra suggesting the presence of floridean starch. The nitrogen supplemented cultures of *G. verrucosa* strain G-16 produced agars of a more polar nature (hot-water soluble) and contained little detectable starch. The data suggest that the higher gelling temperatures of agar from nitrogen limited plants is not due to markedly higher methylation. These data also suggest that nitrogen effects on agar content may not be as significant as previously thought.

Introduction

Agar is one of many commercially important polysaccharides derived primarily from species in the genera *Gelidium*, *Pterocladia* and *Gracilaria*. Recent studies have demonstrated considerable variability with respect to the chemical and physical properties of agar (Cote & Hanisak, 1986; Craigie *et al.*, 1984). Seasonal variability has been documented for such physical properties as gel strength, melting and gelling temperature (Abbott, 1980; Asare, 1980; Hoyle, 1978). Although the basis of seasonal variations remains unclear, culture studies indicate that nutritional status (Bird *et al.*, 1981; Craigie *et al.*, 1984) plays a significant role. Culture studies with *Gracilaria verrucosa* Papenfuss strain G-16 also indicate that nitrogen availability, temperature, and salinity significantly affect the physical properties of agar gels (Bird, 1988; Daugherty & Bird, in press).

Questions remain as to how environmental factors affect the chemical structure of agar or affect the chemical heterogeneity in the populations of agar polymers. Agar type polysaccharides consist of a repeating agarobiose unit of alternating 3-0-β-D-galactopyranosyl and 4-0-3,6-anhydro-α-L-galactopyranosyl sugars. The agarose polymer can be highly substituted with 0-methyl groups, pyruvate ketals or alkali-labile sulfate esters (Araki, 1966; Lahaye *et al.*, 1986), which can alter the physical characteristics of the polymer. For example, gel strength of an agar is inversely relat-

ed to the sulfate content (Rees, 1969) and methylation can increase the gelation temperature (Guiseley, 1970). In this report we examined whether a single environmental parameter (i.e. inorganic nitrogen availability) can significantly affect the heterogeneity of agar polymers in cultured plants. Agar obtained from *G. verrucosa* strain G-16 grown under nitrogen limited or nitrogen enriched conditions was extracted based on its solubility in a variety of aqueous ethanol solutions. Chemical structure of the agar was then characterized by carbon 13 nuclear magnetic resonance spectroscopy.

Materials and methods

The algal material was obtained from a recent culture study which examined the influence of interactive environmental factors on agar quality from *Gracilaria verrucosa* strain G-16. In this study, *G. verrucosa* strain G-16 was grown under controlled culture conditions at two different salinities, two light quantum fluxes, three temperatures and under nitrogen enriched or limited conditions. Subsequent analyses revealed that the most marked differences in agar properties were those between the nitrogen enriched and deficient cultures grown at 32 °C and 33‰ (Bird, 1988; Bird *et al.*, in press). Agar from the nitrogen enriched culture had a higher measurable gel strength (1083 g cm^{-2}) and a lower gelling temperature (42 °C) relative to agar obtained from nitrogen limited conditions (gel strength of 525 g cm^{-2}, gelling temperature of 47 °C). As a result, algae from these two culture conditions were used as a basis for comparing the influence of nitrogen availability on the chemical structural of agar.

Agar polymers were extracted by a modification of the sequential extraction method described by Lahaye *et al.* (1986). Approximately 10 g of dried algal material was ground to a fine power and stirred in 800 ml of acetone (7 h, 24 °C). This extraction was repeated until the acetone supernatants were essentially devoid of insoluble material and color. The algal material was lyophilized and then extracted twice with distilled water to provide a cold water fraction (18 h, 24 °C). Both aqueous supernatants were pooled and stored at 4 °C. The remaining algal material was lyophilized, placed in a round bottom flask fitted with a condenser column (Corning) and sequentially refluxed (1.5 h) in boiling 99.5%, 80%, 60%, 40% and 20% (v/v) aqueous ethanol. After each initial extraction, the refluxed solution was removed and the algal material refluxed with fresh solvent. After 1.5 h, the solvent was removed and pooled with the previous extraction. Following the final ethanol extraction, residual algal material was refluxed in boiling distilled water and then autoclaved in distilled water. All extraction solutions were concentrated with a rotary vacuum apparatus, lyophilized and stored at 4 °C. Total carbohydrate was determined for each extract using the phenol-sulfuric method of DuBois *et al.* (1956); glycogen was used as the standard.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy of agars was performed using 3% (w/v) agar in 50% (v/v) D_2O/H_2O. Prior to analyses, agars were melted in a boiling water bath and sonicated (15 min). Proton composite pulse decoupled 90 MHz carbon 13 spectra were recorded in Fourier Transform mode at 90 °C with a Bruker AM 360 spectrometer (10 mm sample tube, spectral width 12.8 KHz, pulse angle 45°, relaxation decay 1.5 s, 16 K data points). Overnight data accumulations were performed (18,000–20,000 scans). Carbon-13 chemical shifts are expressed in ppm downfield from tetramethylsilane, as referenced to internal dimethylsulfoxide at 39.5 ppm.

Results and discussion

The recovery of total polysaccharides from all ethanol and water extractions was 21.5% and 25.6% per gram dry weight of algal material for the nitrogen enriched and nitrogen deficient cultures, respectively (Figs 1A, B). These recoveries are similar to those found by Lahaye *et al.* (1986) for *Gracilaria blodgettii*, *G. tenuistipitata* and *G. eucheumoides*. The yield of carbohydrate from cold water, 99.5% and 80% aqueous ethanol

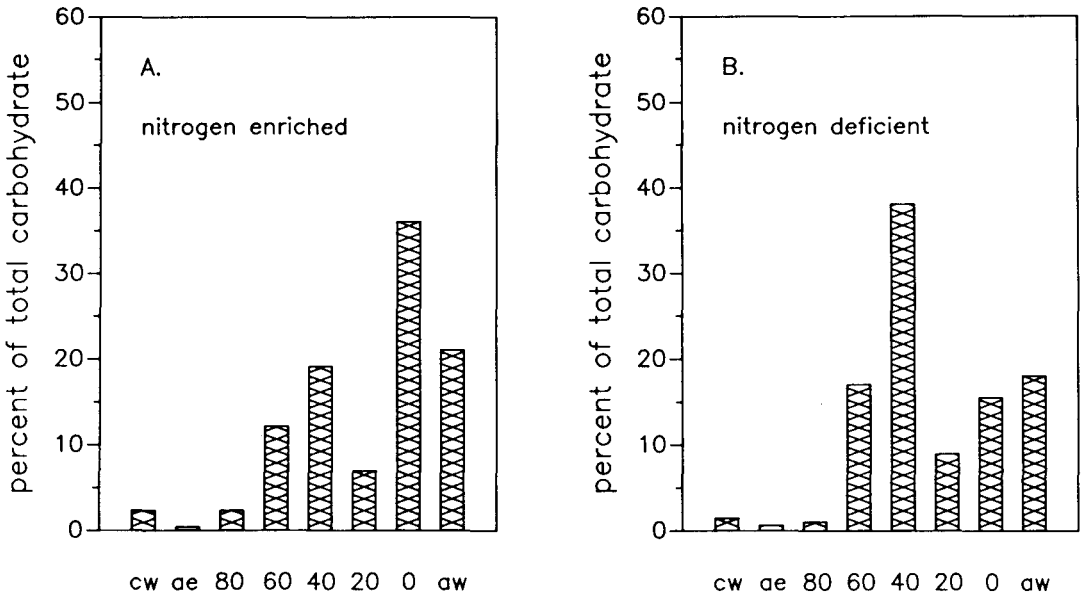

Fig. 1. Recovery of Carbohydrate from *Gracilaria verrucosa* Strain G-16. *G. verrucosa* Strain G-16 was cultured under nitrogen enriched or nitrogen limited conditions. The algae were then extracted with cold water (cw), refluxing with absolute ethanol (ae), 80% (80), 60% (60), 40% (40), 20% (20) ethanol, distilled water (0), or autoclaving in distilled water (aw) as described in Materials and Methods. The yield of carbohydrate in each fraction was determined and is expressed as percent of the total carbohydrate recovered. All data represent the average of three determinations; standard deviations were less than 5% of the mean, except for the nitrogen deficient culture 20%, 0, (aw), which were 15%.

extracts ranged from 5% to 7% of the total carbohydrate recovered from both cultures. Because of the negligible amounts of carbohydrate, these fractions were not further analyzed. There were distinct differences in the recovery of agar polymers from the other fractions. Carbohydrate analyses from nitrogen enriched plants showed the highest recovery in water refluxed and autoclaved extracts, while the profile from nitrogen deficient plants was higher in those fractions extracted in boiling ethanol, particularly 60% and 40% aqueous ethanol. The differences in carbohydrate profiles due to increasing solvent polarity between nitrogen enriched and deficient plants suggest that agar obtained from the latter cultures had a more non-polar nature.

Carbon-13 NMR analysis of these major fractions showed the characteristic signals from the 12 carbons in the basic repeating agarobiose unit, 3-0-β-D-galactopyranose and 4-0-α-L-anhydrogalactopyranose (Figs 2, 3). Carbon-13 NMR spectra signals for each carbon atom in galactose are G1, 102.4; G2, 70.2; G3, 82.2; G4, 68.7; G5, 75.3; G6, 61.4, whereas those for the carbon atoms in anhydrogalactose are A1, 98.2; A2, 69.4; A3, 80.1; A4, 77.3; A5, 75.6; A6, 69.9 (Usov *et al.*, 1983).

Agars extracted from nitrogen limited plants showed several marked differences in ^{13}C NMR spectra from those of nitrogen enriched plants. Carbon-13 NMR spectra of the 60% aqueous ethanol extract, revealed a small, yet discernable signal at 59.1 ppm characteristic of methylation at G6 (Fig. 2A). Due to the low signal to noise, it was difficult to determine if this signal existed in the 60% ethanol extract from the nitrogen enriched material (Fig. 3A). However, in either set of NMR spectra the signals for methylation are considerably less than those reported for other methylated *Gracilaria* agars (Lahaye et al., 1986), suggesting that little methylation is found in the agar from *G. verrucosa* strain G-16. These results suggest that other chemical properties of agar may contribute to the differences observed for the

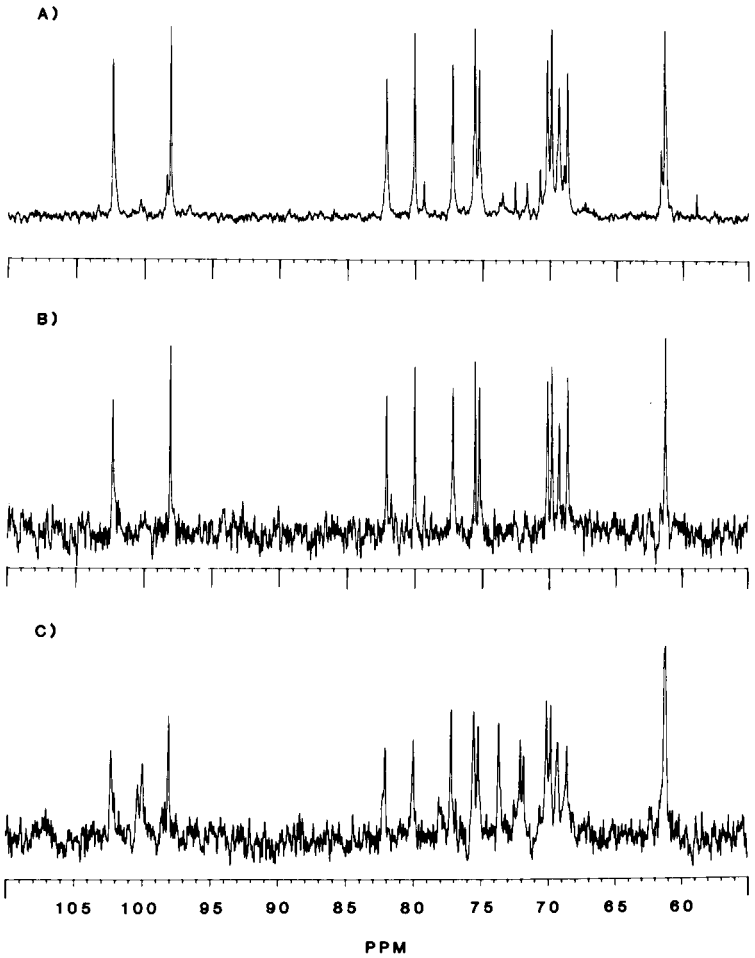

Fig. 2. ^{13}C NMR spectra of agar polymers extracted from *Gracilaria verrucosa* strain G-16 cultured under nitrogen limited conditions. Agar polymers were isolated from *G. verrucosa* strain G-16 by refluxing in 60% (a), 40% (b) and 20% (c) aqueous ethanol.

gelation temperature between these two cultures (42 vs 47 °C). It is noteworthy that Guiseley (1970) also found some variants to the often cited "high gelling temperature – high methylation rule". The causative factors contributing to the nonpolar nature of agar have not been identified; however, they can affect the interactions of the polymers with water in an agar gel, possibly resulting in a reduction of gel strength (Rees, 1972). The differences in gel strength of the alkali pretreated agars from these cultures support this suggestion (Bird, 1988; Bird *et al.*, in press).

The 20% aqueous ethanol and refluxed water extracts from nitrogen deficient algae contained a series of signals at 100.5, 78.1, 73.8, 72.5, and 71.9 ppm (Fig. 2C, data not shown for refluxed water extract), not detected in the 20% aqueous ethanol and refluxed water extracts from nitrogen enriched algal material (Fig. 3C, data not shown for refluxed water extract). These signals probably correspond to floridean starch (Davis & Perlin, 1982) and increase in intensity with increasing solvent polarity. The higher floridean starch content associated with agars from nitrogen deficient plants reopens the question as to whether nitrogen deficiency causes a real increase in agar content (DeBoer, 1979; Bird *et al.*, 1981; Bird, 1988). The differences between gravimetric agar content

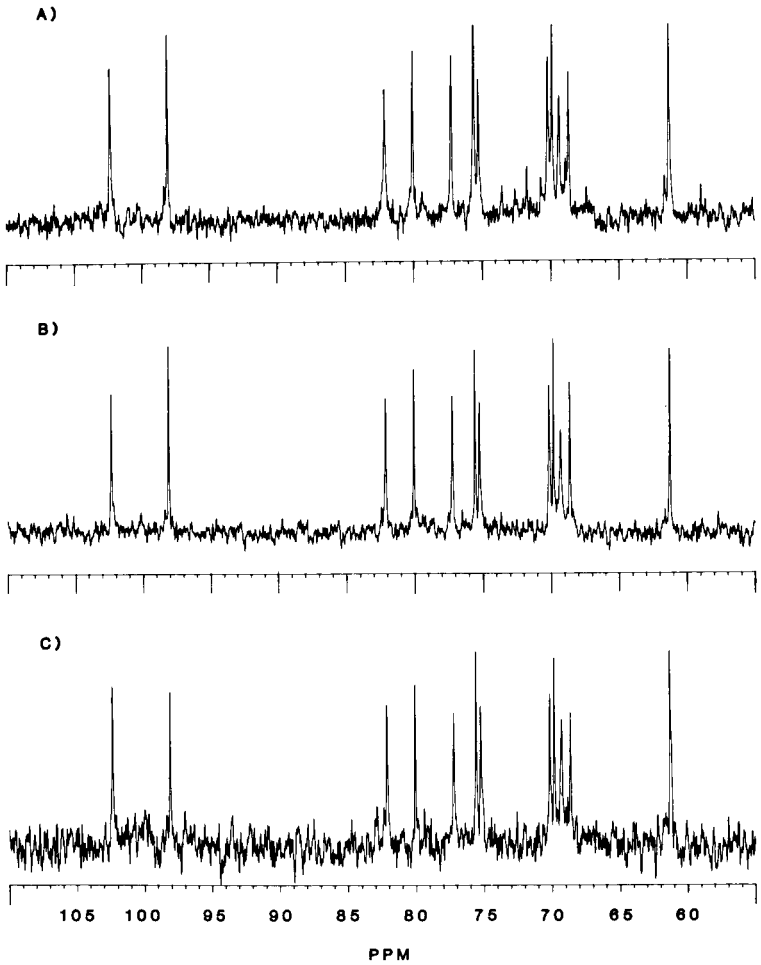

Fig. 3. ^{13}C NMR Spectra of agar polymers extracted from *Gracilaria verrucosa* strain G-16 cultured under nitrogen supplemented conditions. Agar polymers were extracted from *G. verrucosa* Strain G-16 by refluxing the algal material in 60% (a), 40% (b) and 20% (c) aqueous ethanol.

from these two cultures was 20.3% for nitrogen enriched material and 23.2% for nitrogen limited cultures compared to 21.5 and 25.6% based on total carbohydrate, respectively (Bird *et al.*, in press). Because these yields are gravimetric, the differences might be accounted for in part by differences in starch and not just agar content. The presumed relationship between nitrogen limitation and agar yield may not be as significant as thought. The possibility of starch inclusions being partially responsible for these higher gravimetric agar yields has been raised by others (Rotem *et al.*, 1986; Yaphe, personal communication). The proposed mechanisms for biosynthesis of agar and floridean starch suggest that both would be accumulated simultaneously under nitrogen limitation during algal growth (Macler, 1986). It is also interesting to speculate as to whether these starch inclusions are partially responsible for the lower gel strengths of agars extracted from nitrogen limited algae (Bird *et al.*, 1981; Bird, 1988).

There appears to be general differences between carrageenan production by *Chondrus* and agar production by *Gracilaria*. The nitrogen limitation effect on carrageenan content is clearly more significant than the effect on agar content (Neish, 1971; DeBoer, 1979). Furthermore, the chemical structure of carrageenan does not ap-

pear to be as significantly affected by culture conditions.

From an agar production perspective, it is appears that controlled culture conditions could be used to produce agar with more specific kinds of desired chemical structures. This could be accomplished in an aquacultural setting, or perhaps through short term post-harvest culture of a wild harvested crop. The prospects of improving agar quality in wild harvests through such post-harvesting treatments has not been well explored, and may represent a logical combination of resource management and aquaculture.

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