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Short communication

Ontogenic Variation of the Methylation Pattern in Agar-Type Phycocolloids from _Gracilaria verrucosa_ (Huds.) Papenfuss Cultured Under Controlled Conditions: Biotechnological Implications

Mauricio Alfredo Ondarza Beneitez*

Red de Glicociencia en Salud, CONACYT, México

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***Corresponding author:** Mauricio Alfredo Ondarza Beneitez, Red de Glicociencia en Salud, CONACYT, México, E-mail: biochem93@hotmail.com

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Abstract

The influence of tissue age and culture conditions on the chemical composition of galactans extracted from the rhodophyte macroalga _Gracilaria verrucosa_ was evaluated. Using a fractionated extraction procedure by thallus age and alditol acetate derivatization with gas-liquid chromatography (FID), tissue maturation markers were identified. Young tissue (primary lateral branches) showed predominance of 6-O-methyl-D-galactose and a poorly substituted (A-B)n structure, while mature tissue (main axis) showed enrichment in 4-O-methyl-L-galactopyranose. Results suggest that agar is a dynamic polymer and its substitution pattern can be biotechnologically modulated.

Phycocolloids (agar, carrageenans, porphyrans) are linear galactans formed by alternating units (AB) n of D-galactose (Unit A) linked by β(1-3) bond and L-galactose or 3,6-anhydro-L-galactose (Unit B) linked by α(1-4) bond. Their industrial value depends on gel strength, determined by the degree of methylation, sulfation and pyruvation [Craigie and Wen, 1984].

Few studies have addressed the effect of thallus ontogeny on this pattern [Araki et al., 1967].

Introduction

Phycocolloids obtained from red macroalgae are important natural polymers with applications in the food, pharmaceutical, biotechnology, and other industrial sectors. Among these polymers, agar-type galactans are particularly important because their chemical structure and degree of substitution influence their physicochemical and functional properties. Agar consists primarily of alternating galactose residues, with the polymer backbone containing β-(1→3)-linked D-galactopyranose and α-(1→4)-linked L-galactose or 3,6-anhydro-L-galactose residues [1–4].

The chemical properties of agar are influenced by structural modifications including methylation, sulfation, pyruvation, and the occurrence of other substituents. In particular, 6-O-methyl-D-galactose and 4-O-methyl-L-galactose are recognized structural components of agar-type polysaccharides [1,5]. Craigie and Jürgens demonstrated the structural location of 4-O-methyl-L-galactose in agar from *Gracilaria tikvahiae*, providing important evidence for the relationship between methyl substitution and agar structure [5].

The composition and quality of *Gracilaria* agar can vary according to species, physiological condition, life-cycle stage, environmental conditions, culture conditions, and extraction procedures [6–8]. Previous work has also demonstrated that tissue age and environmental factors can influence agar composition and gel properties [7]. Therefore, developmental

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variation in the chemical structure of agar may represent an important factor for understanding and controlling the properties of phycocolloids produced by cultivated red algae.

Structural characterization of agar-type polysaccharides commonly involves methylation analysis, chromatographic techniques, and nuclear magnetic resonance spectroscopy. NMR spectroscopy has been successfully applied to determine the occurrence of methyl groups, sulfate substitutions, 3,6-anhydrogalactose, and other structural features in agar-type polysaccharides [3,4,9]. More recent studies have continued to demonstrate the usefulness of methylation analysis and NMR for determining the structural organization of polysaccharides from *Gracilaria* species [10,11].

Despite the extensive literature concerning the chemical structure and physicochemical properties of agar, comparatively fewer studies have specifically examined the relationship between thallus ontogeny and methylation patterns under controlled culture conditions. The present study therefore investigated different tissues of *Gracilaria verrucosa* representing distinct developmental stages and evaluated the methylation pattern of their agar-type phycocolloids under controlled culture conditions.

Materials and methods

Biological material

Explants of *Gracilaria verrucosa* (Huds.) Papenfuss were separated according to their position and developmental stage within the thallus. The central or main axis, primary lateral branches, and secondary branches were considered representative of tissues differing in ontogenic age.

The use of different tissue portions is relevant because agar yield and quality can vary with physiological and developmental status. Previous studies have demonstrated relationships between environmental conditions, tissue characteristics, and agar quality in cultivated agarophytes [6,7].

Controlled culture conditions

The algal explants were maintained in controlled culture using air-lift glass cylinders. Illumination was provided using Gro-lux and cool-white lamps. The cultures were evaluated under proliferation and non-proliferation conditions.

Controlled cultivation is particularly relevant for examining the influence of physiological conditions on agar composition because growth conditions, including light and nutrient availability, can influence both algal growth and agar characteristics [6].

Extraction and purification of phycocolloids

The agar-type polysaccharides were obtained using a fractionated solubilization procedure. Extraction was performed with water-miscible organic solvents at progressively increasing temperatures to obtain fractions with different solubility characteristics.

The extracted polysaccharide fractions were subsequently purified using ion-exchange chromatography with DEAE-Sephadex A-50 and DEAE-Sepharose CL-6B.

Fractionation and controlled extraction are important in agar characterization because extraction temperature and procedure can influence the composition, molecular characteristics, and functional properties of the recovered polysaccharide [8,12]. Sequential extraction and structural characterization have previously been used successfully for *Gracilaria* agar preparations [8].

Structural characterization

The purified fractions were subjected to alditol acetate derivatization followed by gas-liquid chromatography with flame ionization detection (GLC-FID). This approach was used to determine the monosaccharide composition and identify methylated galactose residues.

Methylation analysis is an established approach for determining glycosidic linkage positions and substitution patterns in red algal polysaccharides [10]. Complementary structural information was obtained using non-destructive ^13C and ^1H nuclear magnetic resonance spectroscopy. NMR has previously been demonstrated to be suitable for identifying methylated residues, 3,6-anhydrogalactose, sulfate substitutions, and other structural features of agar-type polysaccharides [3,4,9].

Results and discussion

Basic structure of the galactan

Structural analyses confirmed that the principal neutral fraction corresponded to agarose, characterized by a galactan backbone containing 3,6-anhydro-α-L-galactose and β-D-galactopyranose residues. This structural organization is consistent with the established molecular architecture of agar-type polysaccharides from red algae [2–4,9].

Agar-type galactans can contain different O-linked substituents, including methyl ether and sulfate groups. The occurrence and distribution of these substituents can influence the physicochemical and gel-forming properties of the polymer [4,9,12].

Effect of tissue ontogeny on methylation

A distinct difference in methylation pattern was observed between tissues representing different stages of thallus development. Young tissues, particularly the primary lateral branches, showed a predominance of 6-O-methyl-D-galactose. This pattern was observed under both proliferation and non-proliferation conditions.

In contrast, mature tissue represented by the main axis showed enrichment in 4-O-methyl-L-galactopyranose, particularly under growth-promoting conditions.

The occurrence of 6-O-methyl-D-galactose and 4-O-methyl-L-galactose is consistent with previously

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described structural features of agar-type polysaccharides [1,5,12]. In particular, Craigie and Jürgens demonstrated the location of 4-O-methyl-L-galactose within agar from *Gracilaria*, supporting the structural interpretation of methylated residues observed in the present study [5].

The observed differences also support the concept that agar is not a chemically static polymer. Instead, the substitution pattern may vary according to the developmental and physiological state of the producing tissue. Earlier work specifically examining tissue age in *Gracilaria* demonstrated that tissue age can influence agar composition and gel strength [7].

Relationship between methylation and metabolic development

The present observations suggest that methylation patterns may be associated with metabolic differentiation during thallus development. Young and mature tissues showed different predominant methylated galactose residues, suggesting that changes in agar structure may accompany developmental changes in the alga.

The precise biochemical mechanism responsible for this variation, however, cannot be established from the present data alone. One possibility is that methylation results from direct anabolic processes involving specific methyltransferase enzymes. Another possibility is that changes in polymer structure involve turnover of existing polysaccharides followed by de novo synthesis of polymers with different substitution patterns.

This interpretation is consistent with the broader understanding that red algal polysaccharides exhibit substantial structural diversity and that their chemical features may be influenced by physiological and environmental conditions [10,11]. Further biochemical investigation, particularly isolation and characterization of enzymes involved in methyl-group transfer, would be required to establish the mechanism.

Influence of structural substitution on agar properties

The functional characteristics of agar are closely associated with its molecular structure. The presence and distribution of methyl and sulfate groups can influence polymer conformation, molecular interactions, and gelation behavior [4,9,12]. Studies of *Gracilaria* agar have demonstrated that structural differences detected by methylation analysis and NMR can be associated with differences in physical properties [8].

Accordingly, the ontogenic differences observed in the present study may have functional consequences for the properties of the extracted hydrocolloid. However, direct measurements of gel strength, viscosity, molecular weight, or rheological behavior were not included in the submitted dataset. Therefore, such relationships should be considered as a potential implication rather than a demonstrated outcome of the present experiment.

Biotechnological implications

The relationship between tissue age, culture conditions, and agar methylation may have practical significance for controlled production of phycocolloids. Previous cultivation studies have demonstrated that environmental conditions can influence growth, agar yield, and agar quality in agarophytes [6]. Similarly, tissue age has been associated with differences in agar composition and gel properties [7].

The present findings suggest that selection of specific developmental tissues may provide an additional strategy for obtaining agar fractions with different structural characteristics. Young explants may be useful when relatively neutral agarose-rich material is desired, whereas mature tissues may provide polymers with greater representation of specific substituted residues.

Recent reviews have highlighted the importance of structural characterization of *Gracilaria* polysaccharides and their potential applications in food, pharmaceutical, and functional-material fields [10,11]. Therefore, controlled cultivation combined with selection of tissue developmental stage may contribute to more predictable production of agar-type hydrocolloids.

Nevertheless, further experiments involving quantitative determination of methylation degree, gel strength, viscosity, molecular weight, and rheological properties are necessary before specific industrial applications can be assigned to the different tissue-derived fractions.

Conclusion

The present study demonstrates an association between thallus ontogeny and the methylation pattern of agar-type phycocolloids from *Gracilaria verrucosa* cultured under controlled conditions. Young tissues were characterized predominantly by 6-O-methyl-D-galactose, whereas mature tissues showed enrichment in 4-O-methyl-L-galactopyranose.

These observations are consistent with established structural studies of agar-type polysaccharides and with previous evidence that tissue age and culture conditions can influence agar composition [5,7]. The findings further suggest that controlled cultivation and selection of specific explant types may provide a potential approach for obtaining agar fractions with distinct structural characteristics.

Future studies should focus on quantitative determination of the degree of methylation, characterization of the relevant methyltransferase enzymes, and assessment of the relationship between methylation pattern and physicochemical properties such as gel strength, viscosity, molecular weight, and thermal behavior.

Biotechnological significance

The identification of ontogeny-associated differences in agar methylation provides a potential basis for controlling

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Figure 1: Controlled cultivation of *Gracilaria verrucosa* under laboratory conditions.
Representative air-lift photobioreactor and associated laboratory setup used for the controlled cultivation of Gracilaria verrucosa *under defined culture conditions.*

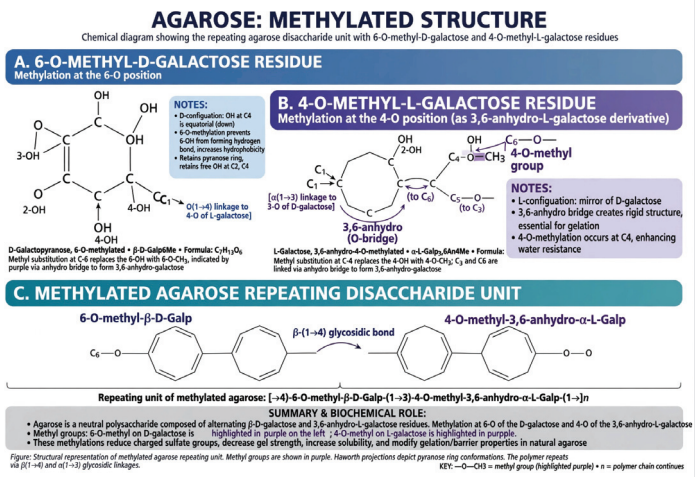


Figure 2: Agarose methylated structure and substituted galactose residues.
(A) 6-O-methyl-D-galactose residue showing methylation at the 6-O position; (B) 4-O-methyl-L-galactose residue showing methylation at the 4-O position; and (C) methylated agarose repeating disaccharide unit illustrating the structural arrangement of the methylated agarose polymer.

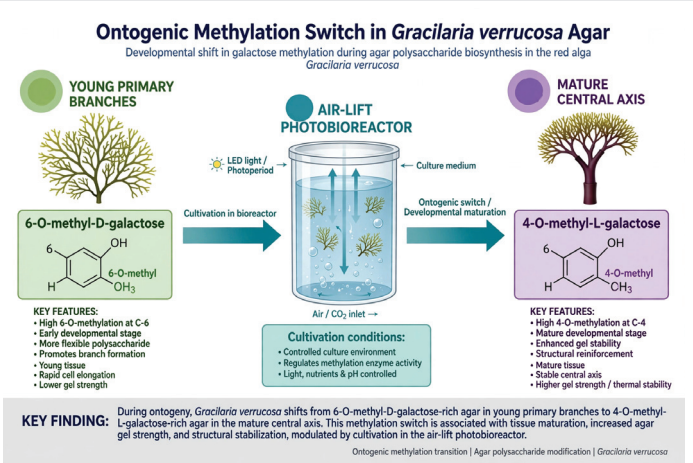


Figure 3: Ontogenic methylation switch in *Gracilaria verrucosa* agar.
Schematic representation of the developmental shift in galactose methylation during agar polysaccharide biosynthesis. Young primary branches are associated with 6-O-methyl-D-galactose-rich agar, whereas the mature central axis is associated with 4-O-methyl-L-galactose-rich agar. The diagram also illustrates controlled cultivation in an air-lift photobioreactor and the proposed transition associated with tissue maturation.

the structural characteristics of phycocolloids produced under cultivation. Because environmental and physiological conditions can affect agar yield and quality [6,7], combining controlled culture conditions with selection of tissue developmental stage may provide a useful strategy for producing agar with more predictable structural characteristics.

The genus *Gracilaria* represents an important source of industrial agar, and recent literature emphasizes its continuing relevance for the development of functional polysaccharide-based materials and products [10,11]. Further optimization of culture parameters and characterization of the biochemical mechanisms underlying methylation will be necessary to translate the present observations into an experimentally validated biotechnological process.

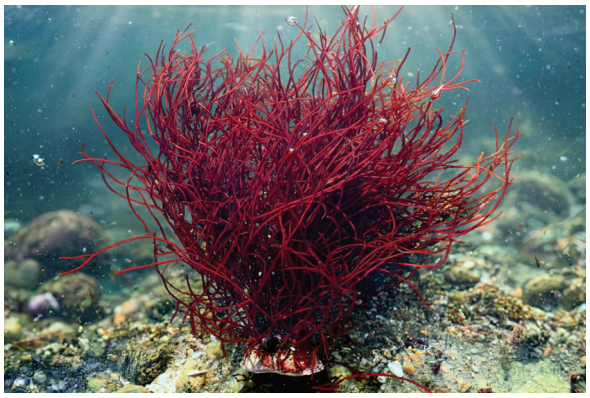


Figure 4: Underwater morphology of *Gracilaria verrucosa*.
Representative underwater image showing the characteristic red, branched thallus morphology of Gracilaria verrucosa.

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