STRUCTURAL CHARACTERIZATION OF AGAR EXTRACTED FROM SIX RED SEAWEED SPECIES GROWING IN THE COAST OF VIETNAM

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ABSTRACT

Polysaccharides extracted from six red seaweed species growing in Vietnam have been studied. Characterisation of their structure by chemical and spectroscopic methods showed that all of them have a basic repeating structure of alternating 3-linked β -D-galactopyranosyl and 4-linked 3,6-anhydro α -L-galactopyranosyl units with substituted methyl ether groups.

The native agar offers only weak gelling abilities owing to the 4-linked α -L-galactopyranosyl - 6-sulfate as its precursor. Conversion of this unit into the corresponding 3,6-anhydride by treating with hot alkali generally led to gel strength increasing.

The agars from *G. fisheri* and *G. firma* are poorly substituted, while those from *G. asiatica*, *G. tenuistipitata* and *G. heteroclada* are partly methylated on position 6 of the 3-linked β -D-galactose. Agar from *Gelidiella acerosa* is partly methylated on both positions 6 and 2 of the 4-linked 3,6-anhydro α -L-galactose.

The alkali- modified agars have been obtained in acceptable quantities with gel strength of 300-932 g/cm². The obtained results showed that all the six algal species are suitable as raw material for commercial agar production and worthy of further cultivation.

Keywords: *Seaweed, Gracilaria, Gelidiella, gel strength, alkali-modified agar*

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1. INTRODUCTION

Agar is a well known polymer originating from two main algal families (*Gracilariaceae* and *Gelidiaceae*). It has a linear backbone made of an alternating 3-linked β -D-galactose (G) and 4-linked 3,6-anhydro L-galactose (LA) units, i.e. agarose, although the latter may be present as a biochemical precursor, i.e. 4-linked α -L-galactopyranosyl 6-sulfate (Fig.1) which can be converted into the corresponding 3,6-anhydride by alkali treatment. The hydroxyl groups in this basic structure are often substituted, mostly with methyl groups on position O-6 of G- or O-2 of LA units. G-unit may contain a pyruvic group 4,6-O [(R)-1-carboxyethylidene] acetal, O-4 or O-6 sulfate esters, and a branching xylopyranose or 4-O methyl L-galactose[1,2].

Fig 1: Structure of idealized agar (A) and agar precursor (B) with common positions of methyl ether substitution.

The gelling properties of agar closely depend on the degree and type of substitution [3, 4], while sulfated residues strongly influence the gel strength and methyl ether groups impact the elasticity and gelling temperature.

In Vietnam five algae among 13 *Gracilaria* species and *Gelidiella acerosa* have been found to be a major source for agar production, that's why they have been widely studied.

The aim of this study was to elucidate chemical structure of the agars extracted from the above mentioned seaweeds to estimate their potential as raw material for commercial agar production as well as for their further cultivation

2. EXPERIMENTAL PROCEDURES

2.1 Materials

Algal samples were air-dried after collection from different sites in Viet Nam coastal. Voucher specimens of the following algal materials used in this study have been deposited in the herbarium of the IMS (Institute of Materials Science), IOH (Institute of Oceanography Hai Phong), ION (Institute of Oceanography Nha Trang): *G. asiatica*, Hai Phong, North Viet Nam, April 2003, IOH 9363; *G. tenuispitata*, Thai Binh, North Viet Nam, January 2003, IMS 203221; *G. heteroclada*, Phu Yen, Central Viet Nam, June 2003, ION 00601; *Gelidiella acerosa*, Central Viet Nam, June 2003, IMS 99510; *G. fisheri*, Kien Giang, South Viet Nam, March 2003, IMS 2002110, *G. firma*, Kien Giang Viet Nam, March 2003, IMS 203290.

2.2 Extraction of native polysaccharides

Samples of air-dried seaweed (40g) were acid treated in 2l of solution 0,11 - 0,18 % H_2SO_4 for 1h, and washed in running tap water for another 2h. The samples were then boiled for 1h with

1,5 l of distilled water (pH 7 - 8) using a Bunsen burner in a 2l Erlenmeyer flask equipped with a reflux condenser. The agar extracts were dehydrated by freezing and pressing.

2.3 Preparation and extraction of alkali-modified agar

Samples of air-dried seaweed (40 g) were treated in 2l of solution 6% NaOH at 70 - 80°C for 3 h, and washed in running tap water for 30 min. The seaweed samples were then extracted as described above for native polysaccharides.

2.4 Gel strength measurements

dried agar (1 g) was placed in a 250 ml beaker, dissolved in distilled water (100 ml) and was pressure cooked for 15 min. The weight of the solution was then adjusted to 100g with additional water. After stirring to ensure that it was homogeneous, the solution was left at room temperature overnight to gel. The beaker was then placed on a top-pan balance, a circular stainless steel rod (crosssection area of 1cm²) was pressed by hand into the gel until it collapsed, and the maximum balance reading was noted. The calculated gel strength was an average of four determinations on the same sample.

2.5 Gel-melting temperature measurements

Agar (30mg), distilled water (3 ml) and a small piece of lead as a weight were placed in a screw-capped culture tube (13 ×100 mm) and pressure-cooked for 20 min (twice if not properly dissolved). After shaking, the solution was allowed to set overnight in a vertical position. A 5 mm glass bead was placed on the gel surface in a glycol bath and heated on hotplate with a temperature rising rate approximately 2°C/min. The gel-melting range was determined as the temperatures of the glycol bath at which the bead started to sink and reached the bottom of the tube. The heating was then stopped, and the tube was allowed to cool. Every minute the tube was removed briefly from the bath and turned horizontally. The gel-setting range was determined as the temperatures between which the solution showed sign of gelling and at which it would no longer flow. All determinations were performed in duplicate.

2.6 Constituent sugar analysis

Alditol acetate derivatives were prepared by a double hydrolysis-reduction procedure [6] and analysed by gas liquid chromatography (GLC) according to the method of Falshaw and Furneaux [7].

2.7 Glycosyl linkage analysis

Samples were converted to a form of triethylammonium salt by dialysis against triethylammonium hydrochloride (0,1 M, pH7, X2), then methylated by the procedure of Stevenson and Furneaux [6] and converted to alditol acetate derivatives for GLC analysis as described above.

2.8 ¹³C-Nuclear magnetic resonance spectroscopy

The ¹³C- NMR spectra were recorded on 5% W/V agar solution, in D₂O-H₂O (1:1) at 80°C on a Bruker AVANCE 500 MHZ.

3. RESULTS AND DISCUSSION

3.1 Agar yield and physical properties

The yield of agar and its gel strength, gelling and melting temperatures from the six red seaweed species are shown in table 1. The yield of native agar (N) from six species of red seaweed varied from 15,5 to 40,0% and the yield of alkali-modified agar (A) from these ones varied at an interval 12,4 - 28,5%.

Treatment of the seaweed samples with hot alkali prior to extraction resulted in a considerable positive effect on gel strength of the agars produced. Gels made from 1% concentration of the alkali-modified agar (A) yielded rather modest strength value of 180 g/cm² for *G. fisheri* and to very strong value 932 g/cm² for *G. tenuispittitata*, meanwhile gel strength of native agar (N), with exception of *Gelidiella acerosa*, ranged 33 -102,9 g/cm². As mentioned, the presence of the precursor units in the corresponding native agar significantly weakens their ability to form gel, that is generally a common observation for agar and carrageenan.

Table 1: Extraction yield and physical properties of the agar specimens.

Species	Sample	Yield %	Agar physical properties		
			Gel strength, g/cm ²	Melting range, °C	Setting range, °C
<i>G. asiatica</i>	N	27,9	91,7	88,5 - 90	42 - 44
	A	28,5	296,0	87,5 - 89,5	42 - 44
<i>G. tenuispittitata</i>	N	15,5	112	86 - 87	39 - 42
	A	17,5	932	88,5 - 90,5	39 - 42
<i>G. heteroclada</i>	N	34,4	60,6	78,5 - 79,5	40 - 41
	A	21,4	637,3	94 - 90	36 - 40
<i>Gel. acerosa</i>	N	37,3	349	94,5 - 95,5	43 - 47
	A	22,6	564	95,5 - 96,5	42 - 46
<i>G. fisheri</i>	N	31,3	33,3	74,5 - 76,5	33 - 36
	A	12,4	180,6	76,5 - 78	35 - 38
<i>G. firma</i>	N	40,0	102,9	88,5 - 91,5	32 - 34
	A	12,5	406,3	92,5 - 94	32 - 34

Gelling temperature of agar extracted from the *Gracilaria* species varied in an interval of 34 - 44°C, while that of *Gelidiella* varied between 42 - 46°C. On the other hand, melting temperature of agar from *Gracilaria* - between 74,5 - 94,5°C, and that from *Gelidiella* ranged from 95 - 98 °C. This agar melted near boiling point of water, about 8 °C higher than other commercial agars.

Gracilaria materials imported from Japan usually provide an agar yield of 14 - 25% which at 1,5% concentration forms a gel strength of 600 - 800 g/cm² and gelling temperature (39-44 °C) [8]. The yield of agar(A) from Vietnamese *G. fisheri* and *G. firma* was not higher than 14% but the yield from the other red seaweeds ranged 17 - 28,2 %. The gel strength value 180-932 g/cm² of the Vietnamese *Gracilaria* agars was lower than that of the Japanese ones. Japanese species, such as *G. chorda*, showed gel strength as high as 1139 g/cm² at 1,5 % concentration [8], while

Gracilaria species from Taiwan, Philippines and Thailand gave the values of 660,45 g/cm², 622,44 g/cm² and 716,14 g/cm², respectively [9].

3.2 Chemical composition

The structure of the alkali-modified agars from six red seaweed species was studied by constituent sugar analysis (table 2), glycosyl linkage analysis (Table 3) and ¹³C NMR spectroscopy (Fig. 2 and table 4).

Methyl ether substitution of agar. Sugar constituent of agar from the red seaweed species are shown in table 2. Three substitution patterns were observed, reflecting the sites of methylation in various species. The agars from *G. fisheri* and *G. firma* were poorly substituted (2 - 4,2 % of 6-O-methyl-D-galactose; 0 - 2,4% of 2-O-methyl 3,6 anhydro-L-galactose), while methylation on G6 (>12%) and little on LA2 were observed for *G. asiatica*, *G. tenuispititata* and *G. heteroclada*. The agar from *Gelidiella acerosa* was methylated on both G6 and LA2. Levels of methylation have also been observed in gel-forming agars from few red algal species, for example, highly 6-O methylated agar from *Euphilota formosissima* (in order *Ceramiales*) [10], 2-O methylated agar from Chinese sample of *G.eucheumoides* [6] and agar methylated on both G6 and LA2 from Japanese *G. eucheumoides* [11]. In general, however, levels of methylation in agar are much lower. So agar from *Gelidium* and *Pteroclada* (as well as few other *Gracilaria*) has less than 10 % methylation on any position. Methylation is not unique to *Gracilaria*, but the presence of substantial methylation (at whatever position) can be used as taxonomic marker for *Gracilaria* species in combination with other characteristic features such as the gross morphology and the presence of tetrasporangial and spermatangial nemathecium [12] . This had already led to the reassessment of the identity of one mislabelled sample.

Table 2: Sugar constituent of Native (N) and alkali-modified (A) agar.

Species	Sample	Constituent (normalised % mol)						Δ Anhydro
		2Me-Angal	Angal	6Me-gal	Gal	Xylose	Gluco	
<i>G.asiatica</i>	A	5,0	38,2	20,6	36,0	-	-	11,0
	N	4,3	28,2	25,0	42,0	0,5	-	
<i>G.tenuispititata</i>	A	-	46,5	12,9	39,9	0,7	-	14,8
	N	-	31,7	16,5	50,6	1,0	-	
<i>G.heteroclada</i>	A	-	42,7	11,9	45,5	-	-	13,7
	N	1,0	28,2	16,2	54,3	-	-	
<i>G.acerosa</i>	A	13,4	34,4	34,4	17,2	-	-	2,0
	N	10,8	35,2	38,6	14,4	1,0	-	
<i>G.fisheri</i>	A	2,4	36,0	3,6	55,0	1,0	2,0	9,6
	N	1,8	27,0	4,2	64,0	1,0	2,0	
<i>G.firma</i>	A	-	42,5	2,0	53,5	1,0	2,0	5,8
	N	-	36,5	2,0	58,5	1,0	2,0	

Precursor residue. In idealized agar structure, the total number of 4-linked 3,6-anhydrogalactopyranosyl unit (determined by constituent sugar analysis as alditol acetate species Angal + 2-MeAngal) should equal the total number of 3-linked galactopyranosyl unit

(determined as Gal + 6-MeGal). A lower level of total 3,6 anhydrogalactopyranosyl unit may indicate the presence of precursor (L6S/L2M6S) unit. Constituent sugar analysis would generate alditol acetate patterns Gal and 2-MeGal from L6S and L2M,6S units, respectively. The level of these precursor unit in the native agar can be estimated from either the decrease in the level of the alditol acetate patterns Gal and/or 6-MeGal (Δ Gal) or the increase in Angal and/or 2-Me Angal (Δ Angal) value in the constituent sugar analyses by going from native to alkali-modified agars once normalised to exclude the variations in level of other sugars [13]. From these values it can be seen that levels of precursor (L6S/L2M,6S) units ranged up to 14,8 mol % (table2).

Table 3: Glycosyl linkage analysis of alkali-modified agar (normalised mol %).

Species	Deduced substitution/linkage								
	4 Angal	2,4 Angal	T-Gal	3 Gal	4 Gal	3,4 Gal	3,6 Gal	3,4,6 Gal	2,3,6 2,4,6 Gal
<i>G.asiatica</i>	46,2	-	-	45,5	3,8	2,0	1,0	1,0	0,5
<i>G.tenuispititata</i>	49,0	-	-	46,0	1,0	3,0	0,5	0,5	-
<i>G.heteroclada</i>	47,5	0,5	-	44,5	2,0	2,5	1,0	1,0	1,0
<i>Gelidiella acerosa</i>	49,0	-	-	45,0	1,0	1,0	1,0	2,0	1,0
<i>G.fisheri</i>	40,0	1,0	3,0	44,0	6,0	1,0	3,0	1,0	1,0
<i>G.firma</i>	46,7	1,0	-	44,0	1,3	1,0	2,0	2,0	1,0

Table 4: ^{13}C NMR spectral assignments (δ , ppm, referred to internal DMSO at 39,47 ppm).

Species	Unit	Carbon shift						
		1	2	3	4	5	6	O-CH ₃
<i>G.asiatica</i>	G	103,06	70,87	82,85	69,37	75,98	62,04	
	G6M	-		-		74,4	72,44	59,71
	LA	98,90	70,5	80,74	77,98	76,27	70,04	
<i>G.tenuispititata</i>	G	103,05	70,87	82,84	69,37	75,98	62,04	
	G6M				69,6	74,2	72,42	59,71
	LA	98,89	70,5	80,74	77,98	76,26	70,02	
<i>G.heteroclada</i>	G	103,06	70,87	82,85	69,37	75,98	62,03	
	G6M					74,22	72,42	59,71
	LA	98,89	70,50	80,73	77,97	76,27	70,02	
<i>Gelidiella acerosa</i>	G	103,09	70,76	82,76	69,30	75,97	62,03	
	G6M				69,6	74,22	72,42	59,71
	LA	98,90	70,49	80,76	78,02	76,26	70,4	
	LA2M	99,37	79,51	79,17				59,83
<i>G.fisheri</i>	G	102,9	70,74	82,72	69,25	75,87	61,91	
	LA	98,81	70,35	80,60	77,87	76,13	69,91	
<i>G.firma</i>	G	103,05	70,87	82,85	69,37	75,97	62,04	
	LA	98,89	70,5	80,73	77,98	76,27	70,03	

¹³C NMR spectroscopy. ¹³C NMR spectroscopy of hot aqueous solutions of alkali-treated agar samples enabled the major structures identified from constituent sugar analysis to be confirmed by comparing the observed chemical shifts (table 4) with literature value for the agars methylated at G6 or LA2 and at both G6 and LA2 positions [14].

The alkali-modified agar from *G. fisheri* and *G. firma* were poorly methyl substituted. Its ¹³C NMR spectrum (fig 2A) revealed 12 major signals corresponding to those of unsubstituted agarose. The constituent analysis of the alkali-modified agar from *G. fisheri* (table 2), however, indicated that the structure may not be as simple as that deduced from ¹³C NMR spectra. As agar was alkali-modified, the total number of 4-linked 3,6-anhydrogalactosyl units should be equal to the total number of 3-linked galactosyl units, hence the expected 1:1 ratio of 3-linked to 4-linked units should be found. However, the calculated ratio was 1,52 :1. The presence of T-gal in the glycosyl linkage analysis data indicated that the galactosyl unit occurs as a single branch of the main agar chain.

Fig. 2: ¹³C NMR of alkali-modified agar from *G. fisheri* (A); *G. heteroclada* (B) and *Gelidiella acerosa* (C).

The alkali-modified agar from *G. asiatica*, *G. heteroclada* and *G. tenuispititata* had a partly methylated agarose. Its ¹³C NMR spectrum (fig 2) contained major characteristic resonances of agarose and minor ones of agarose methylated at O-6 of the 3-linked galactose (Table 4). About 21,7% for *G. tenuispititata*, 31,2 % for *G. asiatica* and 18,5 % for *G. heteroclada* of the 3-linked units were found to be methylated on O-6 position from relative peak area of G6M-5/G5+G6M-5. Constituent sugar analysis of alkali-modified agar revealed nearly equal amount of (Gal+6MeGal) and (Angal) as expected. Glycosyl linkage analysis data of agar from the three

Gracilaria species confirmed the predominance of simple agar of alternating 3-linked β -D-galactopyranosyl and 4-linked 3,6-anhydro α -L-galactopyranosyl units. In addition, the presence 4-galactose confirmed the existence of linked galactopyranosyl units as a main agar chain.

The alkali-modified agar from *Gelidiella acerosa* had highly methylated agarose. Its ^{13}C NMR spectrum (Fig 2C) contained major characteristic resonance of agarose. There are some peaks corresponding to 2-O-Me-3,6-anhydrogalactose (LA2M-1, LA2M-2, LA2M-3) and to 6-Me-galactose (G6M-6, G6M-5, G6M-4). This spectrum also contained a major coalescing signal of the two methoxyl carbons appeared as almost one pick at 59,71 and 59,83 ppm, which would be attributed to 6-O-methyl-D-galactose and 2-O-methyl-3,6-anhydrogalactose. The data indicated that agar isolated from *Gelidiella acerosa* consists of two kind of methylated sugars. About 61 % of the 3-linked units were found to be methylated on O-6 and about 35,8 % of the 4-linked units - on O-2 positions (on the basis of relative peak areas of LA2M-1/LA2M-1 + LA2M-1). Glycosyl linkage analysis data of the alkali-modified agar from *Gelidiella acerosa* also confirmed the predominance of a simple agar structure of alternating 3-linked β -D-galactopyranosyl and 4-linked-3,6-anhydrogalactopyranosyl units.

4. CONCLUSION

Polysaccharides extracted from six red seaweed species growing in Vietnam were structurally investigated using constituent sugar and glycosyl linkage analyses, and ^{13}C -NMR spectroscopy. Characterisation of their structure by chemical and spectroscopic methods showed that all of them have a basic repeating structure of alternating 3-linked β -D-galactopyranosyl and 4-linked 3,6-anhydro α -L-galactopyranosyl units with substituted methyl ether groups.

The experimental data showed that agars from *Gracilaria fisheri* and *G. firma* were poorly substituted, while for *G. asiatica*, *G. tenuistipitata*, *G. heteroclada* and *Gelidiella acerosa* the methylation (on G6) was considerable.

For the alkali-modified agars from *G. asiatica*, *G. heteroclada* and *G. tenuistipitata* the ^{13}C NMR spectra demonstrated major characteristic resonances of agarose and minor ones of agarose methylated at O-6 of the 3-linked galactose. The glycosyl linkage analysis data of agar from the three *Gracilaria* species confirmed the predominance of simple agar with alternating 3-linked β -D-galactopyranosyl and 4-linked 3,6-anhydro α -L-galactopyranosyl units.

For the alkali-modified agar from *Gelidiella acerosa* the ^{13}C NMR spectra also showed major characteristic resonances corresponding to 2-O-Me-3,6-anhydrogalactose (LA2M-1, LA2M-2, LA2M-3) and to 6-Me-galactose (G6M-6, G6M-5, G6M-4). This agar consists of two kind of methylated sugars, where 61 % of the 3-linked units were found to be methylated on O-6 and about 35,8 % of the 4-linked units - methylated on O-2 position. The glycosyl linkage analysis data of alkali-modified agar from *Gelidiella acerosa* also confirmed the predominance of a simple agar structure with alternating 3-linked β -D-galactopyranosyl and 4-linked-3,6-anhydrogalactopyranosyl units.

The obtained results indicated that all the six algal species are suitable as raw material for commercial agar production and worthy of further cultivation.

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REFERENCES

1. Craigie, J.S. (1990), Cell wals. In: (K.M. and R. G. Sheath. Eds), Cambridge Univercity Press, pp. 221-257.
2. Furneaux, R.H., Miller, I.J., and Svenson, T.T. (1990), *Hydrobiologia*, 204/205, pp. 645-654.
3. Whyte, J.N.C, Englar, J.R., Sauders, R.G., and Linsay, J.C. (1981), *Bot. Mar.* 24, pp. 493-501.
4. Lahay, M., Rochas, C., and Yaphe, W. (1985), *Can. J. Bot.* 64, pp. 579-585.
5. Nguyen, H.D. (1992), In I. A. abbot (ed), 3, pp. 207-210.
6. Steven, T.T, and Furneux, R.H. (1992), *Carbohydr. Res.* 210, pp. 277-298.
7. Falshaw, R. and Furneux, R.H. (1994), *Carbohydr. Res.* 252, pp. 171-182
8. Orosco, C.A., Chirapart, A., Nukaya, M., Ohno, M., and Sawamura, M., *Nippon Gakkaishi* (1992), 58(9), pp. 1771-1716.
9. Tam, D.M. and Edwards, P. (1982), *Bot. Mar.* 25, pp. 456-465.
10. Miller, I.J. and Furneaux, R.H. (1997), *Bot. Marina* , 40, pp. 333-339.
11. Takano, R., Hayashi, K., and Hara, S. (1995), *Phytochemistry*, 40, pp. 487-490.
12. Nelson, W.A. and Knight, G.A. (1997), *Newzealand J. Bot.* 35, pp. 195-202.
13. Hemmingson, J.A. and Muray-Brown, V.H. (1997), *Carbohydr. Res.* 287, pp. 101-115.
14. Minghou, J., Lahaye, M., and Yaphe, W. (1985), *Bot. Marina*, 28, pp. 521-528.
15. Knutsen, S.H, Myslabodski, D.E., Larsen, B., and Usov, A.I. (1994), *Bot. Marine*, 37, pp. 163-169.