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THE AGAR COMPONENT OF THE RED SEAWEED *GELIDIUM PURPURASCENS*

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Abstract—Chemical composition and rheological properties of agar isolated from *Gelidium purpurascens*, the agar after alkaline treatment, and a commercial agar are presented. The agar and alkali-treated agar gave weaker gels, as measured with an Instron 1122, than those of commercial agar. Xylose, glucose and glucuronic acid in the agar were removed together with 86% of the nitrogen content on alkaline treatment, indicating occurrence of these residues as carbohydrate–protein complexes. Sequential extraction of the alga accounted for low yields of agar as losses incurred on ethanol precipitation. Acid treatment of the residue from exhaustive aqueous extraction of the alga liberated a further 10% agar with increased gel strengths despite increased glucose inclusion, suggesting a lack of involvement of these 'contaminant' carbohydrate–protein residues in helix coil formation during gelling.

INTRODUCTION

The ubiquitous genus *Gelidium* (Rhodophyceae) constitutes *ca* 35% of the world's annual exploitation of agarophytes, estimated at 39000 tonnes, with over 40 species being harvested throughout the world as raw material for the 6500 tonnes of agar produced annually [1, 2]. Agar consists of 3-linked-β-D-galactopyranose residues alternating with 4-linked 3,6-anhydro-α-L-galactopyranose residues in a repeating linear sequence. However this structure is frequently masked to varying extents by having the D-galactose residues bearing either methoxyl, sulphate hemi-ester, or 4,6-*O*-(1'-carboxyethylidene) groups and the anhydro-L-galactose residue replaced by L-galactose-6-sulphate or substituted by methoxyl groups [3]. This structure would encompass most of the types of polymers elaborated by agarophytes amongst which are the Gelidiales. *Gelidium purpurascens* (Gardner) is one of the principal members of this order on the coast of British Columbia and in this paper the isolation, composition and rheological properties of the constituent agar is described.

RESULTS AND DISCUSSION

Gelidium purpurascens on freeze-drying yielded 26.1% dry solids of which 17.3% was total ash and 3.4% insoluble ash. Hydrophobic and low MW hydrophilic constituents were extracted with chloroform–methanol–water [4], leaving a 78.3% residue consisting principally of proteinaceous and carbohydrate polymers. Determination of nitrogen, 2.13% in this residue, allowed an accurate assessment of protein, 10.4% in the dry alga, by virtue of the removal of associated low MW nitrogenous compounds on extraction.

Extraction of the dry alga with hot water yielded 15.7% agar which on hydrolysis followed by PC (solvents A, B and C) was shown to contain principally galactose with minor amounts of xylose, 6-*O*-methylgalactose, glucose and a trace of glucuronic acid. Except for glucose, these minor sugars have been reported to be associated with the agar molecule, however, following alkaline borohydride treatment of the polymer [3] xylose, glucose and glucuronic acid residues were absent from the products of hydrolysis. Sugars in the hydrolysates were quantified by GC of the corresponding alditol acetates [5] using mannitol as the internal standard (Table 1). Xylitol acetate and 6-*O*-methylgalactitol acetate cochromatographed on column (b), RR_t mannitol acetate, 0.75, but were completely resolved on column (a), RR_t mannitol acetate, 0.66 and 0.57, respectively. A film of the polysaccharide isolated exhibited a characteristic infrared spectrum for agar [6] with the presence of 3,6-anhydrogalactose demonstrated by the absorption at 940 cm^{-1}. Composition and properties of the agar before and after alkaline treatment together with pharmaceutical grade agar (BBL lot 504604) are presented in Table 1.

Chemical compositions of the untreated agar from *G. purpurascens* and the commercial agar were similar (Table 1) even to the minor inclusions of xylose and glucose. That these sugars and the trace of glucuronic acid, detected by PC, were not directly incorporated into the agar molecules was suggested by their removal on base treatment. Significantly an 86% decrease in nitrogen content was also effected by this treatment (Table 1) implying that these sugars in the agar were present as carbohydrate–protein contaminants.

The highest content of 6-*O*-methylgalactose correlated well with the highest value for gelation temperature of the alkali-treated agar and was in accord with the observation that increasing methoxyl content results in increasing gelation temperatures of agars [7]. Gel melting temperature is considered dependent on the MW of the polymer [8] thus the 7–9° differential in this parameter between agar from the alga and the commercial agar is consistent with the marked difference in viscosity (Table

Table 1. Chemical composition and properties of the untreated and alkali-treated agar from *G. purpurascens* compared with commercial agar

Parameters	Untreated agar	Alkali-treated agar	Commerical agar
Anhydrogalactose, %	32.6	42.3	35.5
Galactose, %	59.7	46.7	53.8
6-*O*-Methylgalactose, %	1.5	2.2	1.5
Xylose, %	1.8	nil	0.7
Glucose, %	2.6	nil	1.1
Sulphate, %	2.7	1.0	2.2
Nitrogen, %	0.91	0.13	0.15
Gelation temp.,	35.4	39.2	34.9
Gel melting temp.	94.4	92.6	85.0
Viscosity, cP	64.6	140	1.9
Gel strength, g cm^2	89.8	175	247
Gel deformation, mm	4.3	4.0	3.0
Gel cohesion, mm	3.0	2.7	2.0
Gel rigidity, g cm^2/mm	14.9	32.8	62.0

1). These values, as comparable measures of MW, would suggest that the mild isolation procedure used on the alga and the upgrading by alkaline treatment resulted in higher MW agar than that isolated from the more rigorous commercial extraction methods [9].

In general, the 3,6-anhydrogalactose component presents a measure of gel strength with firmer gels being provided by the agarose molecules consisting of 1,3-linked-β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose residues, the latter forming 47% of the idealised structure by weight. Where the L-galactose residues are substituted at the 6-position with sulphate ester groups, the formation of the corresponding anhydrosugar can be effected by alkali treatment [3]. Thus the agar from *G. purpurascens* following alkaline treatment increased in anhydrogalactose content to 42%, decreased in sulphate content to 1% and exhibited an enhanced gel strength (Table 1) which was measured from force deformation plots, recorded by an Instron Universal Testing Machine. The resulting two-fold increase in gel strength was also accompanied by declines in gel deformation and cohesion values indicating a less elastic, more brittle gel having an increased degree of rigidity (Table 1).

Numerous instruments and devices have been used to determine gel properties of agars and carrageenans, consequently a lack of uniformity exists in the data presented in the literature. An objective evaluation of the agar from *G. purpurascens* was based therefore on a direct comparison with values obtained from quality bacteriological grade agar under standardized testing conditions (Table 1). Agar from *G. purpurascens*, as isolated and even after alkaline treatment, proved inferior in gel strength to this commercial product and provided an elastic gel more suited to food industry uses. As gelation involves association of chains of double helices having all but the 2-hydroxyl group of the D-galactose residue pointing outwards from the helix cavity [10], partial substitution at this position by residual sulphate even after alkaline treatment would account for the inferior gel by preventing normal coiling of the agar. No direct correlation appeared to exist between the viscosity measurements and the strength of the corresponding gels (Table 1), suggesting that gel strength of the agar was dependent on the extent of concurrent unsubstituted sugar units, primary agarose blocks, within the molecules rather than the size of the molecules.

Yields of agar vary with species of alga, season, location and environment and commercially 17–25% yields from agarophytes is considered normal [11]. The 16% yield of agar from *G. purpurascens* undoubtedly reflected some of these variables yet the 78% polymeric material in the alga known to contain 13.3% protein appeared contradictory to the low yield for agar and warranted an explanation.

Accountability for the agar in another sample of *G. purpurascens*, indicated that major losses occurred in the isolation of the polymer by addition of ethanol despite the observed apparent completeness of precipitation. Incomplete precipitation of lower MW polymers was indicated by viscosity measurements of 33 and 84 cP for agar A and Ap respectively. Increases in the gel strength of the agar B and Bp isolated after acid treatment and in the ethanol precipitated agars Ap and Bp (Table 2), suggested increases in agarose blocks in these fractionated polymers.

That residual agar was associated with cellulosic and proteinaceous components was illustrated by the 4.5% galactose and 7.8% glucose in the hydrolysate of the exhaustively extracted residue III, the latter sugar being derived from minor degradation of the β(1–4)-linked glucan [3]. The 31.1% protein in residue III accounted for 86.5% of the total protein in the alga.

Analysis of the agar hydrolysates (Table 2) illustrated the preferential removal on ethanol precipitation of the more hydrophobic 6-*O*-methylgalactose containing agar molecules, together with an increase in the glucose content of the agars following acid treatment of the alga. As cellulose fragments were removed from the agar solutions by high speed centrifugation, this increase in glucose was attributed, as indicated previously, to carbohydrate–protein contaminants. Although the inclusion of the glucose increased on fractionation of the

Table 2. Composition of acid hydrolysates and properties of the agar fractions isolated before and after acid treatment

Properties	Agar A	Agar Ap	Agar B	Agar Bp
Galactose, %	42.2	45.7	40.6	30.6
6-*O*-Methylgalactose, %	1.6	1.3	2.4	1.4
Xylose, %	1.4	1.3	1.1	1.7
Glucose, %	3.5	3.2	9.7	11.3
Gel strength, g/cm^2	24.5	38.7	124	185
Gel deformation, mm	3.8	3.7	4.4	4.7
Gel cohesion, mm	2.6	2.5	3.0	3.3
Gel rigidity, g/cm^2/mm	4.8	7.8	20.8	28.1

agar (Table 2) no adverse effects on gelation characteristics were evident, suggesting these carbohydrate–protein contaminants had little influence on gel formation.

EXPERIMENTAL

Algal material. Gelidium purpurascens (Gardner) was collected on 17 September 1977 at Bamfield Inlet, Vancouver Island and stored as freeze-dried material.

General methods. PC on Whatman No. 1 was carried out with the following solvents: (A) EtOAc–HOAc–HCO_2H–H_2O (18:3:1:4), (B) EtOAc–pyridine–H_2O (10:4:3), (C) *n*-BuOH–EtOH–H_2O (3:1:1). Sugars were detected with *p*-anisidine HCl [12] or alkaline $AgNO_3$ [13]. Acid hydrolysis of agars (25 mg) and algal material (50 mg) employed 1 N H_2SO_4 at 100° for 16 hr followed by neutralization with $BaCO_3$. Sugars in the hydrolysates were quantified as corresponding alditol acetates [5] by GC on columns (1.8 m × 0.32 cm) of (a) 5% Silar 10C on Gas Chrom Q (100–120 mesh) operating at 200° with a N_2 flow rate of 30 ml/min and (b) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) programmed from 150° (4 min) to 185° (hold) at 2°/min, with N_2 flow rate of 30 ml/min. Mannitol (1 mg) was added to the reduced hydrolysate, prior to acetylation, as int. standard. Total ash was assessed by ignition of samples of dry alga at 500° for 20 hr, and the insoluble ash was determined by measuring residual material after hot H_2O leaching of an ashed sample. Polymeric material in the alga was assessed from the residue after exhaustive extraction with $CHCl_3$–MeOH–H_2O (2:4:1) [4]. N_2 was determined by the micro-Kjeldahl method. Anhydro-3,6-anhydrogalactose content was determined by reaction with resorcinol according to the method of ref. [14] except for the modified use of synthesized methyl 3,6-anhydro-α-D-galactose [15] as the standard sugar for calibration purposes. Sulphate was determined spectrophotometrically using 4-amino-4′-chlorodiphenyl reagent [16]. IR spectra were determined on polysaccharide films.

Isolation of agar. Dry alga (30 g) was washed with H_2O (6 × 4 l., 21°) to remove excess salts then extracted with H_2O (1.5 l.) at 95–100° for 1.5 hr. The resultant mixture was centrifuged while hot at 7000 rpm for 15 min and the residue extracted with a further 1.5 l. H_2O at 95–100°. A third extraction with H_2O (1 l.) for 0.5 hr yielded only minor quantities of agar. The combined centrifugate was evapd to 1 l. and the agar precipitated with 5 vol. EtOH. Purification involved dissolution of the polymer in H_2O (600 ml), dialysis and precipitation with EtOH. For ease of handling, the agar was redissolved in H_2O and freeze-dried (4.71 g).

Alkaline treatment of agar. Agar (2 g), dissolved in boiling H_2O (200 ml), was cooled and maintained at 50° while $NaBH_4$ (20 mg) was added. The soln was stirred for 45 min prior to the addition of NaOH (50%, 12 ml). After stirring for a further 2.5 hr at 50° the soln was neutralized carefully with 1 N HCl and the modified agar precipitated with EtOH (2 l.). The polymer was washed with EtOH prior to dissolution in H_2O (400 ml) and on evapn to 200 ml was allowed to gel. Purification by two freeze–thaw cycles provided the alkali-treated agar (1.12 g).

Isolation of agar before and after acid treatment. Dry algal material (28 g) was extracted (× 4) with $CHCl_3$–MeOH–H_2O (2:4:1, 600 ml) [4] to provide residual material which was dried under high vacuum (21.7 g; N, 2.05%). This polymeric residue I was extracted (× 4) with H_2O (1 l.) at 95–100° for 1 hr. Centrifugation at 6000 rpm from 20 min provided a residue which was freeze-dried to afford polymeric residue II (11.69 g). After reheating the centrifugate to 95°, it was clarified by passage through a Sharples centrifuge, concd and freeze-dried to yield crude agar A (10.01 g). Following dissolution in H_2O the agar was dialysed overnight against running H_2O then freeze-dried to yield agar fraction A (8.88 g). A portion (5 g) of agar A was dissolved in H_2O (500 ml) and 5 vol. EtOH added to provide a ppt. which was subsequently freeze-dried to afford precipitated agar, Ap (2.77 g).

Acid treatment of the polymeric residue II (11.69 g) was effected by suspension in H_2O (1.7 l.) and stirring at 21° for 24 hr at pH 5, using 1 N H_2SO_4 for pH control. Stirring was continued for a further 6 hr at 80° with checks and adjustments to pH 5 being made when necessary. On cooling to 21°, the mixture was neutralized with 0.1 N NaOH and the agar extracted as described previously. Centrifugate was clarified by passage through a Sharples centrifuge then dialysed and freeze-dried to afford agar fraction B (2.77 g). A portion of agar B (1.60 g) was dissolved in H_2O (160 ml) then 5 vol. EtOH added to provide precipitated agar Bp (0.75 g) which was freeze-dried. The polymeric residue III (7.74 g; N, 4.97%) was freeze-dried.

Physical analysis of agars. All rheological properties of the agars were measured in aq. soln. (1%, w/w). Gelation temp. was determined by the periodic introduction of glass beads (3 mm dia.) to a controlled cooling soln of the agar and the temp. recorded when the bead just failed to sink. Gel mp of the agar soln was recorded when beads placed just below the surface of the resultant gel, sank into the test soln heated at 1°/min. Viscosity in centipoise (cP) of the 1% soln was measured at 65° with a Brookfield Viscometer Model LVF using the No. 1 or No. 2 spindle.

Texture profile analysis was performed on the 1% aq. soln, 30 ml of which was poured while hot into each of 3 50 ml beakers

(42 × 53 mm) and allowed to gel and equilibrate overnight at room temp. with the beakers covered by Al foil. Testing with an Instron Universal Testing Machine Model 1122, operating at a crosshead speed of 10 mm/min, a chart speed of 50 mm/min, with a 1 cm^2 stainless steel probe, provided values for gel strength, deformation, cohesion and rigidity of the gels. The load–deformation curve provided rheological parameters which were defined as follows. Gel strength was the max load required to rupture the gel matrix in the immediate vicinity of the applied load (g/cm^2). Deformation was the total distance penetrated by the plunger into the gel matrix prior to surface rupture (mm). Cohesion was the deformation of the gel surface which corresponded to half the rupture load (mm). Rigidity was defined as the load per unit distance required to deform the surface of the gel which was calculated from the slope of the first half of the load–deformation curve, providing a measure of the firmness or stiffness of gel network (g/cm^2/mm). Average reproducibility for all rheological parameters was within $\pm 2.0\%$.

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REFERENCES

1. Yamada, N. (1976) *J. Fish. Res. Board Can.* **33,** 1024.
2. Levring, T., Hoppe, H. A. and Schmid, O. J. (1969) *Marine Algae. A Survey of Research and Utilization.* Cram, de Guyter, Hamburg.
3. Mackie, W. and Preston, R. D. (1974) *Algal Physiology and Biochemistry* (Stewart, W. D. P., ed.) p. 40. Blackwell Scientific, Oxford.
4. Whyte, J. N. C. and Southcott, B. A. (1970) *Phytochemistry* **9,** 1159.
5. Sawardeker, J. S., Sloneker, J. H. and Jeanes, A. (1965) *Analyt. Chem.* **37,** 1602.
6. Tsuchiya, Y. and Hong, K. C. (1966) *Proc. Int. Seaweed Symp.* **5,** 315.
7. Guiseley, K. B. (1970) *Carbohydr. Res.* **13,** 247.
8. Selby, H. H. and Wynne, W. H. (1973) *Industrial Gums, Polysaccharides and their Derivatives* (Whistler, R. L., ed.) p. 29. Academic Press, New York.
9. Okazaki, A. (1971) *Seaweeds and their Uses in Japan* p. 112. Tokai University Press.
10. Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C. M., Moorehouse, R. and Rees, D. A. (1974) *J. Mol. Biol.* **90,** 269.
11. Moss, J. R. (1977) *The Marine Plant Biomass of the Pacific Northwest Coast* (Krauss, R. W., ed.) p. 309. Oregon State University Press.
12. Hough, L., Jones, J. K. N. and Wadman, W. H. (1950) *J. Chem. Soc.* 1702.
13. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. (1950) *Nature* **166,** 444.
14. Yaphe, W. (1960) *Analyt. Chem.* **32,** 1327.
15. Lewis, B. A., Smith, F. and Stephens, A. M. (1963) *Methods in Carbohydrate Chemistry* 2 (Whistler, R. L. and Wolfrom, M. L., eds.) p. 172. Academic Press, New York.
16. Jones, A. S. and Letham, D. S. (1956) *Analyst* **81,** 15.