THE AGAR POLYSACCHARIDES OF *Gracilaria* SPECIES*

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ABSTRACT

Polysaccharides from *Gracilaria debilis*, *G. compressa*, *G. foliifera*, *G. domingensis*, *G. damaecornis*, and *G. ferox* have been evaluated as sources of agar. Chemical and enzymic analyses, coupled with fractionation of the agars on DEAE Sephadex A-50, have shown the differences in the series of related polysaccharides which constitute different agars. Of these agars, only *G. debilis* agar has a high gel-strength. The gel strength of the agars from *G. debilis*, *G. compressa*, and *G. foliifera* are increased by alkaline treatment. Possible reasons for the differences in gel strengths of the agars are discussed.

INTRODUCTION

Agar is a complex mixture of polysaccharides extracted from species of the red algae known as the agarophytes. On acetylation, the agar from *Gelidium amansii* yields two fractions[1], a virtually neutral polymer, agarose, and a charged fraction, agaropectin. Araki[2,3] deduced the structure of agarose to be (1→4)-linked 3,6-anhydro-α-L-galactose alternating with (1→3)-linked β-D-galactose. Agaropectin is thought to have the same repeating unit as agarose, although some of the 3,6-anhydro-L-galactose residues can be replaced with L-galactose sulphate[4] residues and there is partial replacement of the D-galactose residues with the pyruvic acid acetal 4,6-*O*-(1-carboxyethylidene)-D-galactose[5]. Recent fractionation studies[6] on DEAE Sephadex A-50 have indicated that agar is not made up of one neutral and one charged polysaccharide but is composed of a complex series of related polysaccharides which range from a virtually neutral molecule to a highly charged galactan. These studies indicated that, in agars containing 4,6-*O*-(1-carboxyethylidene)-D-galactose, this charged residue is always found in regions of the molecule that are low in sulphate. Agars in which the D-galactose residues can be partially replaced with 6-*O*-methyl-D-galactose contain this 6-*O*-methylated monosaccharide evenly distributed throughout the neutral and charged regions of the complex[4].

It is well-established that an agar having a high gel-strength can be obtained

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from a *Gracilaria* species[4], but agars from only a few species have been extensively studied. The gelling property of an agar is due to the three equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues, which constrain the molecule so as to form a helix; interaction of helices causes gel formation[7]. Replacement of 3,6-anhydro-L-galactose residues with L-galactose sulphate causes kinks in the helix, and hence a polysaccharide of lower gel-strength is formed. If these sulphate groups are at C-6, they can be converted into 3,6-anhydro-L-galactose by treatment[8] with alkali, and an increase in gel strength results. The presence of 6-*O*-methyl-D-galactose does not appear to affect gel strength, but the extent of 6-*O*-methylation does affect the gelling temperature[9].

The current study on *Gracilaria* agars is part of a programme to evaluate the agars from different groups of agarophytes.

RESULTS

Chemical analysis. — The major products after complete hydrolysis of the polysaccharides with acid are D-galactose, 6-*O*-methyl-D-galactose, 5-hydroxymethyl-2-furaldehyde, and a trace of xylose. The hydrolysates from *Gracilaria foliifera*, *G. damaecornis*, *G. domingensis*, and *G. ferox* agars also contain a sugar having a chromatographic mobility slightly less than that of 6-*O*-methyl-D-galactose and the same electrophoretic mobility in borate buffer as 4-*O*-methyl-L-galactose. Araki has reported that both methyl sugars are present in commerical agars[4].

Quantitative determinations were carried out before and after treatment with alkali (Table I). The molar ratio of D-galactose plus 6-*O*-methyl-D-galactose to 3,6-anhydro-L-galactose plus sulphate is *ca.* 1:1. The content of 6-*O*-methyl-D-galactose varies from 2.3% for the polysaccharide from *G. compressa* to 21.0% for that from *G. foliifera*. After treatment with alkali, there is an increase in the content of 3,6-anhydro-L-galactose (except for *G. domingensis* agar, the 3,6-anhydro-L-galactose content of which remains constant), a decrease in D-galactose (the reason for which is unknown), and no significant change in the content of 6-*O*-methyl-D-galactose. The amount of pyruvic acid liberated from the agars by hydrolysis with dilute acid [which is indicative of the 4,6-*O*-(1-carboxyethylidene)-D-galactose content] varies from 0.1% for *G. ferox* agar to 2.92% for *G. compressa* agar. The pyruvic acid content is not affected by treatment with alkali.

Table I shows the relationship between 6-*O*-methyl-D-galactose, D-galactose, sulphate, and pyruvic acid in the *Gracilaria* agars studied. The highest content of pyruvic acid occurs in agars having a sulphate content of 3.5–4.5%[10] and a low content of 6-*O*-methyl-D-galactose. Low values of pyruvic acid favour a high content of 6-*O*-methyl-D-galactose. These relationships may be explained if we consider that, during biosynthesis, the 4,6-acetal is added at the polysaccharide level. Previous results[6] have shown that 4,6-*O*-(1-carboxyethylidene)-D-galactose is found in regions of the molecule having a low content of sulphate, and therefore the D-galactose residues available for the formation of this 4,6-acetal will depend on the extent of sulphation and 6-*O*-methylation.

Before treatment with alkali, only the agar from *G. debilis* (sulphate, 3.4%) has a high gel-strength. After treatment with alkali, the polysaccharides can be divided into three groups. The polysaccharides in group 1 (from *G. debilis* and *G. foliifera*) have high gel-strengths and low contents of sulphate and ash. The *G. compressa* polysaccharide in group 2 has intermediate values. The polysaccharides in group 3 (from *G. ferox*, *G. damaecornis*, and *G. domingensis*) have low gel-strengths and high contents of sulphate and ash.

An agar polysaccharide only gels when the sulphate content of a sufficient percentage of the whole complex is so low that the number of kinks in the molecule, due to the difference in the conformation of L-galactose sulphate and 3,6-anhydro-L-galactose, are at a minimum[7]. The difference in gel strength between *G. debilis* and *G. foliifera* agars is therefore due either to a more-random distribution of the sulphates in *G. foliifera*, or because the higher concentration of 6-*O*-methyl-D-galactose in *G. foliifera* decreases its gel strength. The analytical data (of the alkali-treated polysaccharides) indicate that, for a non-gelling agar polysaccharide, the sulphate content must approach ~1% on treatment with alkali before gelling occurs. *G. compressa* agar (1.3% of sulphate after treatment with alkali) does not gel because of the effect of the high content of 4,6-*O*-(1-carboxyethylidene)-D-galactose (2.92%). No direct evidence has been obtained on the position of the sulphate groups in any of these polysaccharides, but the resistance of the sulphate groups of the agars from *G. ferox*, *G. damaecornis*, and *G. domingensis* on treatment with alkali indicates that most of the sulphate is not located at C-6. The high ratios of L-:D-galactose residues (when all the sulphate is assumed to be attached to L-galactose residues) for the agars from *G. ferox*, *G. damaecornis*, and *G. domingensis* indicate that some of the D-galactose residues could be sulphated.

The relationship between gel strength and structure is clarified by fractionation of the agars on DEAE Sephadex A-50 (Fig. 1). The polysaccharide eluted with water has a high gel-strength and approaches the structure of an ideal agarose. Thus, the percentage of the total polysaccharide eluted in this fraction will indicate the gelling properties of the unfractionated agar.

The agar from *G. debilis* is the only polysaccharide capable of forming a firm gel before treatment with alkali, and it is the only one that has a significant amount of polysaccharide eluted in the water fraction. *G. foliifera* agar, which has a lower content of sulphate, has a smaller proportion of polysaccharide eluted in the water fraction, and this agar therefore forms a weaker gel. It seems, therefore, that it is the arrangement of sulphate groups, rather than the extent of 6-*O*-methylation, which prevents the *G. foliifera* agar from forming a firmer gel. This is confirmed by the fact that, on treatment of the agar from *G. foliifera* with alkali, the sulphate content is only decreased from 2.5 to 1.1%, the content of 6-*O*-methyl-D-galactose does not change appreciably, and yet there is a marked change in gel strength (20→220 g).

Of the agars examined, that from *G. compressa* forms the third strongest gel after treatment with alkali. On fractionation with DEAE Sephadex A-50, the bulk of the polysaccharide is eluted with 0.5M sodium chloride. This is characteristic of a

TABLE I

CHEMICAL COMPOSITION OF THE *Gracilaria* AGARS

Alga	*Gel strength* (g)	D-*Galactose*[a] (%)	6-O-*Methyl*[a]-D-*galactose* (%)	*Total* D-*galactose* (%)	*Pyruvic acid* (%)	*3,6-Anhydro*[a]-L-*galactose* (%)	*Sulphate* (SO_4^{2-}, %)	*Total* L-*galactose*[b] (%)	*Molar ratio* L:D
BEFORE TREATMENT WITH ALKALI									
G. ferox	30	28.6	12.0	40.6	0.10	36.4	7.0	50.4	1.24
G. damaecornis	10	30.5	12.2	42.7	0.17	35.6	6.0	47.6	1.11
G. domingensis	14	36.5	9.6	46.1	0.52	37.8	6.0	49.8	1.08
G. compressa	41	41.2	2.3	43.5	2.92	37.0	4.1	45.2	1.04
G. debilis	140	37.2	8.4	45.6	0.18	36.1	3.4	42.9	0.94
G. follifera	20	22.9	19.6	42.5	0.13	37.6	2.5	42.6	1.00
AFTER TREATMENT WITH ALKALI									
G. ferox	40	22.0	12.5	34.5	0.10	39.8	5.5	50.8	1.47
G. damaecornis	50	23.7	13.0	36.7	0.15	37.5	5.5	48.5	1.29
G. domingensis	22	26.7	13.7	39.9	0.45	37.8	4.7	47.2	1.18
G. compressa	80	35.6	2.9	38.5	2.50	40.6	1.3	43.2	1.12
G. debilis	335	32.7	10.2	42.9	0.12	47.3	0.8	48.9	1.14
G. follifera	220	18.0	21.0	39.0	0.10	40.8	1.1	43.0	1.10

[a]Expressed as a percentage of the polysaccharide. [b]All the sulphate is assumed to be on the L residues for the purpose of this calculation.

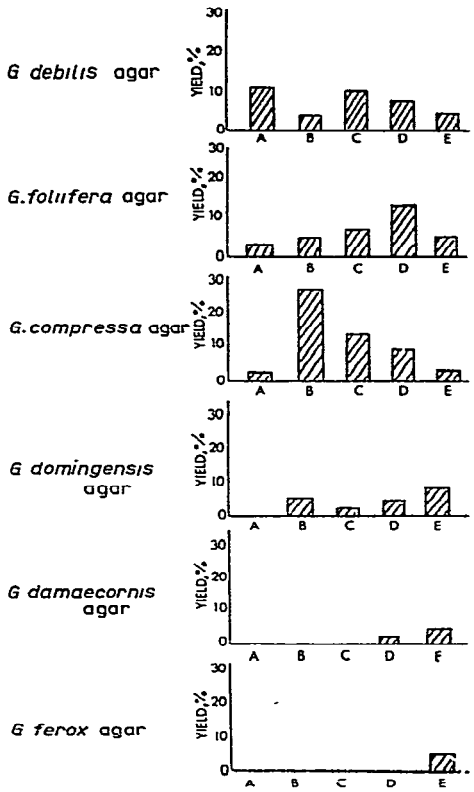

Fig. 1. Bar diagrams showing the fractionation of *Gracilaria* agars on DEAE Sephadex A-50 (Cl^-) The polysaccharide was eluted from the ion exchanger with water (A) and 0.5M (B), M (C), 2.0M (D), and 3 0M (E) sodium chloride.

polysaccharide having a high 4,6-*O*-(1-carboxyethylidene)-D-galactose content[10]. *G. compressa* agar therefore forms a weak gel, because the majority of molecules approach the structure of "pyruvated agarose" instead of the ideal, neutral agarose. *G. domingensis*, *G. damaecornis*, and *G. ferox* agars form the weakest gels, and it is therefore not surprising to find that these polysaccharides are strongly adsorbed to DEAE Sephadex A-50 and are only partially eluted with 3M sodium chloride.

From these fractionations, it is concluded that the agars studied are a closely related series of polysaccharides, the gel strength of which decreases as the extent of masking of the basic agarose structure increases. The anomaly of *G. debilis* agar and *G. foliifera* agar is discussed above.

None of the *Gracilaria* agars examined had a gel strength which approaches that of *Gelidium cartilagineum* or *Gelidium sesquipedale* agars (both approximately 300 g). These *Gelidium* agars are known to be commercial sources of agar and agarose. The usefulness of these *Gracilaria* agars is therefore limited to the weaker gels required by the food industry.

Enzymic analysis. — All known agarases[3,11,12] degrade agar by cleaving the β-(1→4) linkage between D-galactose and 3,6-anhydro-L-galactose to form oligosaccharides based on the disaccharide neoagarobiose. An extracellular agarase from a *Cytophaga* species has been used to obtain structural information on the related polysaccharides agarose, porphyran, and the galactan sulphate from *Laurencia pinnatifida*[13,14]. More recently, the agarase from *Pseudomonas atlantica* has been used to obtain structural details on the agar from *Gelidium cartilagineum*[15].

Previous results[16,17] have shown that it is difficult to interpret the cellulose thin-layer chromatograms of enzymic hydrolysates of *Gracilaria* agars, by using one- or two-dimensional chromatography. The neutral and charged oligosaccharides must first be separated from one another on DEAE Sephadex A-25 (Cl^-), followed by desalting on Sephadex G-10 and then separate studies in various solvent systems.

The t.l.c. separation of the neutral and charged oligosaccharides and the electrophoretograms of the charged oligosaccharides at pH 2.0 and 6.0 were compared. It was found that all the *Gracilaria* agars examined gave the same pattern of neutral oligosaccharides (R_{Gal} 1.7, 1.3, 1.1, and 0.8) and a trace of an oligosaccharide with R_{Gal} 1.5 (solvent *A*, double development). The quantity of each oligosaccharide differed with the source of the agar. By comparison with the neutral oligosaccharides produced by the enzymic degradation of porphyran[14], the four main oligosaccharides were deduced to be 6^1-*O*-methylneoagarobiose, neoagarobiose, 6^3-*O*-methylneoagarotetraose, and neoagarotetraose.

On electrophoresis of the charged oligosaccharides, only those from *G. domingensis* and *G. compressa* agars contained oligosaccharides which lost their mobility at pH 2.0. The M_S values for the mixture of sulphated oligosaccharides were the same for each agar hydrolysate (M_S 1.13, 1.02, 0.89, 0.75, 0.58, 0.49, and 0.30), but no evidence was obtained to indicate whether each spot represents a single oligosaccharide or mixtures of oligosaccharides having the same charge-to-mass ratio. The charged oligosaccharides from the different agars also gave very similar patterns when studied by t.l.c. (solvent *C*).

The products formed are the same in each case but the ratios differ. The agars having a high content of 6-*O*-methyl-D-galactose, such as *G. foliifera* agar, yield more 6-*O*-methylated oligosaccharides, whereas those having a high content of pyruvic acid yield more oligosaccharides containing 4,6-*O*-(1-carboxyethylidene)-D-galactose. Similarly, the ratio of neutral to charged oligosaccharides ranges from 82:18 for *G. foliifera* agar to 12:88 for *G. ferox* agar.

These results confirm the previous conclusion that the *Gracilaria* agars consist of a closely related series of polysaccharides, in which the extent of masking varies.

Information on the basic repeating unit of the different agars may be obtained by incubating the polysaccharides with a mixture of agarase and β-neoagarotetraosase. The agarase cleaves internal β-(1→4) linkages between D-galactose and 3,6-anhydro-L-galactose in a random manner, yielding, as final products, neoagarotetraose and neoagarobiose. If the agar contains 6-*O*-methyl-D-galactose, the products will also contain 6^1-*O*-methylneoagarobiose and 6^3-*O*-methylneoagarotetraose, and if 4,6-*O*-

(1-carboxyethylidene)-D-galactose is present, the enzymic hydrolysate will contain $4^3,6^3$-*O*-(1-carboxyethylidene)neoagarotetraose. The β-neoagarotetraosase[18] hydrolyses neoagarotetraose to two moles of neoagarobiose per mole, and specificity studies have shown that this enzyme can also hydrolyse 6^3-*O*-methylneoagarotetraose but not $4^3,6^3$-*O*-(1-carboxyethylidene)neoagarotetraose.

Consequently, the final, neutral products on enzymic hydrolysis (agarase plus β-neoagarotetraosase) of the *Gracilaria* agars are the two disaccharides neoagarobiose and 6^1-*O*-methylneoagarobiose, the ratio of which varies with the ratio of D-galactose to 6-*O*-methyl-D-galactose. The polysaccharides from *G. compressa* and *G. domingensis*, on degradation with agarase plus β-neoagarotetraosase, also produced $4^3,6^3$-*O*-(1-carboxyethylidene)neoagarotetraose. The agarase produces a series of sulphated oligosaccharides of high molecular weight from all the agars, which were not degraded further by the β-neoagarotetraosase.

The analytical data, fractionation on DEAE Sephadex A-50, treatment with alkali, and enzymic degradation, when used in conjunction, are valuable in evaluating the structure and usefulness of an agar. One of the techniques taken alone provides limited information which could be misleading. It was hoped, at the outset of this work, that the enzymic technique alone would be able to evaluate the agars with respect to agarose content, but it is limited in that it does not take into account the arrangement of the charged sugars. For example, the enzymic degradation of the agar from *G. foliifera* produces 82% of neutral oligosaccharides, but the agar is still not capable of forming a firm gel.

EXPERIMENTAL

Collection of Gracilaria *species.* — *G. damaecornis*, *G. ferox*, *G. domingensis*, *G. compressa*, and *G. debilis* were collected in the inter-tidal and intra-littoral waters of the Caribbean island of Barbados during June 1967. Following collection, the material was sorted, air-dried, and placed in plastic bags for shipment to Montreal. The sixth species, *G. foliifera*, was collected at Pomquet harbour, Nova Scotia, in February 1966, milled to a powder, and stored at −20°.

Preparation of polysaccharides. — Air-dried *Gracilaria* (50 g) was extracted with distilled water (1.5 l) for 3 h at 100°. The extract was filtered and allowed to gel. The gel was cut into strips and frozen at −10°. The frozen polysaccharide was thawed, filtered, dehydrated with ethanol, and dried *in vacuo* at 45°.

Modification of the polysaccharides with alkali was carried out by the method described by Rees[8].

Analytical methods. — 6-*O*-Methyl-D-galactose was determined quantitatively by the method of Wilson[19], D-galactose with D-galactose oxidase[20], and 3,6-anhydro-L-galactose by the method of Yaphe and Arsenault[21]. Sulphate was determined by the method of Jones and Letham[22], and pyruvic acid by the lactate dehydrogenase method[23]. Gel strength was measured with a semi-microgelometer[24] on a 2% gel which had aged overnight at 5°. A plunger of 8-mm diameter was used at a speed of

compression of 0.36 mm/sec. The agarase[25] and β-neoagarotetraosase[18] were prepared as already described.

Chromatography. — Paper chromatography was carried out with butyl alcohol–acetic acid–water (4:1:2), and the sugars were detected with aniline hydrogen phthalate[19].

The enzymic hydrolysates were not examined by chromotography until the neutral and charged oligosaccharides had been separated on DEAE Sephadex A-25 (Cl^-). The neutral oligosaccharides were eluted from this ion-exchanger with distilled water, and the charged with 2M sodium chloride. Both the neutral and charged oligosaccharides were desalted on Sephadex G-10 (fine) before being studied by cellulose t.l.c. (Camag microcrystalline cellulose). The neutral oligosaccharides were separated by the solvent systems (*A*) butyl alcohol–pyridine–water (2:1:1) or (*B*) butyl alcohol–ethanol–water (3:2:2). The charged oligosaccharides were best separated by the solvent system (*C*) butyl alcohol–acetic acid–water (4:1:2). The oligosaccharides were detected with the naphthoresorcinol reagent[17].

Electrophoresis. — Paper electrophoresis was performed on Schleicher and Schuell AG No. 2043A paper in 0.1M sodium tetraborate (pH 9.2) at 1250 volts for 2 h, or pyridine–acetic acid buffer (pH 6.1) at 3000–4000 volts for 20 min.

Polysaccharide fractionation. — The agars were fractionated on DEAE Sephadex A-50 (Cl^-) by using conditions which have been described previously[6].

Enzyme hydrolysis. — The agars were hydrolysed by using the conditions which have been described previously[15].

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