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Chemical and macromolecular characterisation of agar polymers from *Gracilaria dura* (C. Agardh) J. Agardh (Gracilariaceae, Rhodophyta)

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Agar polymers were extracted with and without alkali pre-treatment from the red seaweed *Gracilaria dura*, growing in the northern Adriatic Sea. Chemical analysis combined with ^{1}H and ^{13}C NMR spectroscopy showed that agar polysaccharides characterised by low methoxyl and fairly high pyruvate contents were obtained. Alkali pre-treatment markedly reduced the molecular weight and significantly increased the polydispersity of the extracted polymers. The native and alkali-treated agars exhibited comparable gelling and melting temperatures; however, the alkali-treated sample had higher gel strength than that of the native one. Chemical structure, molecular weight and rheological properties of the extracted galactans are discussed with regard to the algal source.

INTRODUCTION

Carbohydrate polymers extracted from seaweeds are currently used for biological and industrial applications (Lewis *et al.*, 1988; Skjåk-Bræk & Martinsen, 1991). Among these polymers, agar extracted from the cell wall of certain species of Gelidiaceae and Gracilariaceae play increasingly important roles in various areas of emerging biotechnology (Renn, 1984). Agar is a mixture of water-soluble galactan derivatives and is commercially important because of its gel-forming properties. It is generally agreed that the basic repeat unit of this polysaccharide is agarobiose which consists of 4-*O*-3,6-anhydro-*α*-L-galactopyranose and 3-*O*-*β*-D-galactopyranose (Araki & Arai, 1956). However, a variable amount of substituent groups are present on the polysaccharide chain; the most frequent substituents are methoxyl, sulphate esters and pyruvate ketal groups. Moreover, L-galactose-6-sulphate residues are often present as a partial replacement of 3,6-anhydro-L-galactose (Hirase, 1957; Araki & Hirase, 1960; Duckworth & Yaphe, 1971; Izumi, 1972; Painter, 1983).

The type and amount of substituents depends on the algal species and strain (Craigie *et al.*, 1984; Lahaye, 1986; Levy & Friedlander, 1990), on various environmental and physiological factors (Hoyle, 1978; Asare, 1980; Whyte *et al.*, 1981; Craigie & Wen, 1984; Lahaye & Yaphe, 1988) and on the procedures used in extracting and isolating agar (Rees, 1961; Craigie & Leigh, 1978). Quite variable contents of 6-*O*-methyl-D-galactose and 2-*O*-methyl-3,6-anhydro-L-galactose are found in *Gracilaria* spp. Methylation of the 3-linked residue is widespread and a high methoxyl content

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(37%) was discovered in *G. tikvahiae* (Craigie *et al.*, 1984). Different degrees of methylation of the 4-linked residue are also found for several species and a complete substitution was reported for *G. eucheumoides* (Ji Minghou *et al.*, 1985). In addition 4-*O*-methyl-L-galactose may be present in significant quantities in agars from some species (Craigie *et al.*, 1984; Karamanos *et al.*, 1989). On the other hand, pyruvic acid, bound as 4,6-*O*-(1-carboxyethylidene)-D-galactose (Painter, 1983), is not always detectable in agar polysaccharides from *Gracilaria* spp. However, a relatively high amount of pyruvate (2·92%) was revealed in *G. compressa* (Craigie, 1990). Alkali-stable and alkali-labile sulphates may occur in different positions of the disaccharide unit. In the case of *Gracilaria* spp., alkaline hydrolysis of sulphate, with conversion of the L-galactose-6-sulphate into 3,6-anhydro-L-galactose, is often employed to improve the gel strength of the agar obtained (Armisen & Galatas, 1987).

The gel-forming properties of agar are widely influenced by the chemical substituents (Guiseley, 1970; Tagawa & Kojima, 1972), as well as by the molecular weight and molecular weight distribution of the agar polymers (Watase & Nishinari, 1983). However, the role of the molecular weight in the gelling mechanism of agar has not been extensively investigated.

The purpose of the present work is to determine the chemical structure and the physical properties of the agar extracted from *Gracilaria dura*, collected in the northern Adriatic Sea. In particular, attention is paid to the changes in chemical structure and molecular weight caused by alkali pre-treatment. In addition, a new method for the determination of molecular weight and molecular weight distribution of agar polymers is also described.

EXPERIMENTAL

Agar sources

The agar polymers investigated were obtained from specimens of *Gracilaria dura* growing in the northern Adriatic Sea. Thalli of this species, identified by Prof. G. Tripodi of the University of Messina (Italy), were collected in December in the Bay of Sistiana (Trieste, Italy). Agarose 'standard EEO' (11400) from Serva, used without any further purification, was chosen for comparison because of its very low sulphate content and high gel strength.

Extraction and purification

After collection, specimens were washed with tap water, cleaned from impurities and dried. Typically, 4 grams of dry alga were alkali treated with 25 ml of 0·5N aqueous NaOH at 90 °C under nitrogen for 3 h. The mixture was cooled to 40 °C, neutralised with equimolar HCl, washed thoroughly with cold water and then extracted with 250 ml of degassed, distilled water at 90 °C under a nitrogen atmosphere. After 30 min, the hot solution was successively filtered at 70 °C through 2·7 and 1·2 μm glass microfibre filters (Whatman GF/D and GF/C). Extraction of agar without alkali pre-treatment was also performed, giving a product which in the following is referred to as native polymer.

To break down floridean starch, the hot solutions were treated with amylase from *Bacillus licheniformis* (Termamyl 120 L, Novo Industri A/S, Denmark) at 70 °C for 1 h. Agar polymers were recovered from solutions by means of precipitation with isopropyl alcohol and centrifugation at 7000 rev. min^{-1} for 10 min. Pellets were subsequently washed twice with 75% isopropyl alcohol, resuspended into a small volume of distilled water and dialysed exhaustively against distilled water. Finally the agar was redissolved in distilled water at 90 °C, filtered through 0·7 μm glass microfibre filters at 70 °C (Whatman GF/F) and freeze-dried.

Chemical analysis

The content of 3,6-anhydro-L-galactose was determined colorimetrically by using a resorcinol assay (Yaphe & Arsenault, 1965), with fructose as the standard sugar.

Sulphur content was quantified by elemental analysis of the powdered specimen by using a Carlo Erba elemental analyser (model EA 1108) coupled with an electron capture detector. From the weight per cent of sulphur, the content of sulphate was expressed as moles of ester per galactose residue ($C_6H_{10}O_5$).

NMR spectroscopy

^1H and ^{13}C NMR spectra of 1–3% (w/v) agar solutions in D_2O (99·9%) were acquired on a Bruker AC 200 NMR spectrometer, equipped with a variable temperature unit and a $^1H/^{13}C$ dual probehead for 5 mm diameter sample tubes. ^1H NMR spectra (200·13 MHz) were recorded at 80 °C, after dissolution at 90 °C, using the water elimination Fourier transform (WEFT) technique (Gupta, 1976) to suppress the residual HOD signal. ^1H chemical shifts were measured in parts per million from internal sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propionate (TSP). Proton composite pulse decoupled ^{13}C NMR spectra (50·33 MHz) were recorded overnight at 80 °C and the chemical shifts were referred to tetramethylsilane (TMS) by setting the internal dimethylsulphoxide to 39·6 ppm.

The methoxyl and pyruvic acid ketal content was determined by ^1H NMR spectroscopy, using pre-saturation of the residual HOD signal. The degree of methylation was calculated as previously described by

Lahaye *et al.* (1988). The pyruvate content was estimated according to the method of Kennedy *et al.* (1984), using quinol (Aldrich-Chemie) as the internal standard.

Molecular weights

The determination of the molecular weight averages and of the molecular weight distribution curves was carried out by means of a high performance gel permeation chromatography (HPGPC) system previously described (Martinsen *et al.*, 1991). The signals were detected using an LDC-Chromatix CMX-100 low angle laser light scattering (LALLS) photometer (equipped with a He-Ne laser, λ_0 = 632·8 nm) and a Waters 410 differential refractometer. The column set (Waters μBondagel E-High Å, E-1000, E-500, E-125) was thermosttated at 60 °C. A 0·5 μm post-column filter (FHUP Millipore) was present in all HPGPC-LALLS experiments. Owing to the strong tendency of agar macromolecules to associate in aqueous solvents, all the samples were dissolved at 60 °C in dimethyl sulphoxide and 0·1M tetrabutyl ammonium bromide (TBABr). The polymer concentration was 2 gL^{-1} and the solutions were filtered through a 0·5 μm pore size membrane (FHUP Millipore) before injection. The refractive index (RI) and LALLS signals were analysed by a PCLALLS™ (Ver. B, LDC Milton Roy) software for the absolute determination of the molecular weight. The RI signal was also processed by means of the Chromstar data acquisition system (Ver. 2·07, Bruker Spectrospin) for the relative GPC determination. In order to evaluate the exact polymer concentration of the extracts of *G. dura* after filtration, a calibration curve was obtained by integrating the RI signal of the commercial sample. The refractive index increment (dn/dc = 0·06 mL g^{-1}) determined for this sample in a KMX 16 differential refractometer (LDC Chromatix) at 632·8 nm was then used for measurements of the other samples.

Rheological analysis

The gel strength was measured using a Stevens-LFRA texture analyser. Hot agar solutions (1·5% w/v in 0·005M $NaHCO_3$) were poured into plastic cylinders (14·8 mm diameter, 15 mm height) closed at the bottom by a dialysis membrane and allowed to gel overnight at 25 °C in a Petri dish covered with solvent. The gel strength measurements were done in triplicate and the values expressed as g cm^{-2}.

Melting temperatures were determined in triplicate by heating 1·5% (w/v) agar gels with a temperature increase of about 0·5 °C per min and recording the temperature at which the glass bead placed on top of each gel sank.

Gelling temperatures were measured in triplicate by cooling 1·5% (w/v) hot agar solutions with a temperature drop of about 0·5 °C per min. A thermometer, placed in the agar solution, was periodically withdrawn and the temperature at which a permanent depression or hole remained was considered as the gelation temperature.

RESULTS

The total yield of polymer was 27·7 and 21·2% for the native and alkali-treated agar, respectively, on a dry-weight basis. Chemical analysis showed that the native agar from *Gracilaria dura* had a high sulphur content when compared with the commercial sample. However, the alkali pre-treatment significantly reduced the molar ratio of sulphur to sugar residue from 0·025 to 0·016 and enhanced the weight percentage of 3,6-anhydrogalactose up to 43·9 (Table 1).

NMR spectroscopy

NMR spectroscopy confirmed that agarobiose was the main repeating unit in the polysaccharides extracted from *G. dura* according to published assignments (Welti, 1977; Usov *et al.*, 1980). In addition, a direct comparison of spectra of the native and alkali-treated agars from *G. dura* with those of the commercial agarose showed that the extracted polymers were characterised by fairly high contents of pyruvate and low degrees of methylation (Figs 1 and 2).

The sharp signal at 1·47 ppm in the ^1H NMR spectra of the extracts from *G. dura* (Fig. 1(b) and (c)) was assigned to the methyl group of pyruvic acid ketal linked to the 4- and 6-positions of β-D-galactopyranose residues (Izumi, 1973). Moreover, the low signal at 5·24 ppm was attributed to the anomeric proton of the L-galactopyranose residue, being deshielded by the adjacent pyruvate substituent. The quantitation of pyruvate from integration of the signal at 1·47 ppm

Table 1. Chemical analysis of the commercial agarose and agar polymers extracted from *Gracilaria dura*

Sample	Quantity of sulphate ester (mol/$C_6H_{10}O_5$)	3,6-Anhydro-L-galactose (% w/w)	Pyruvate (% w/w)	2-*O*-Me-L-galactose (DS)	6-*O*-Me-D-galactose (DS)
Commercial	0·004	48·8	n.d.	0·16	0·72
Native	0·025	38·1	1·2	0·04	0·09
Alkali treated	0·016	43·9	1·7	0·02	0·06

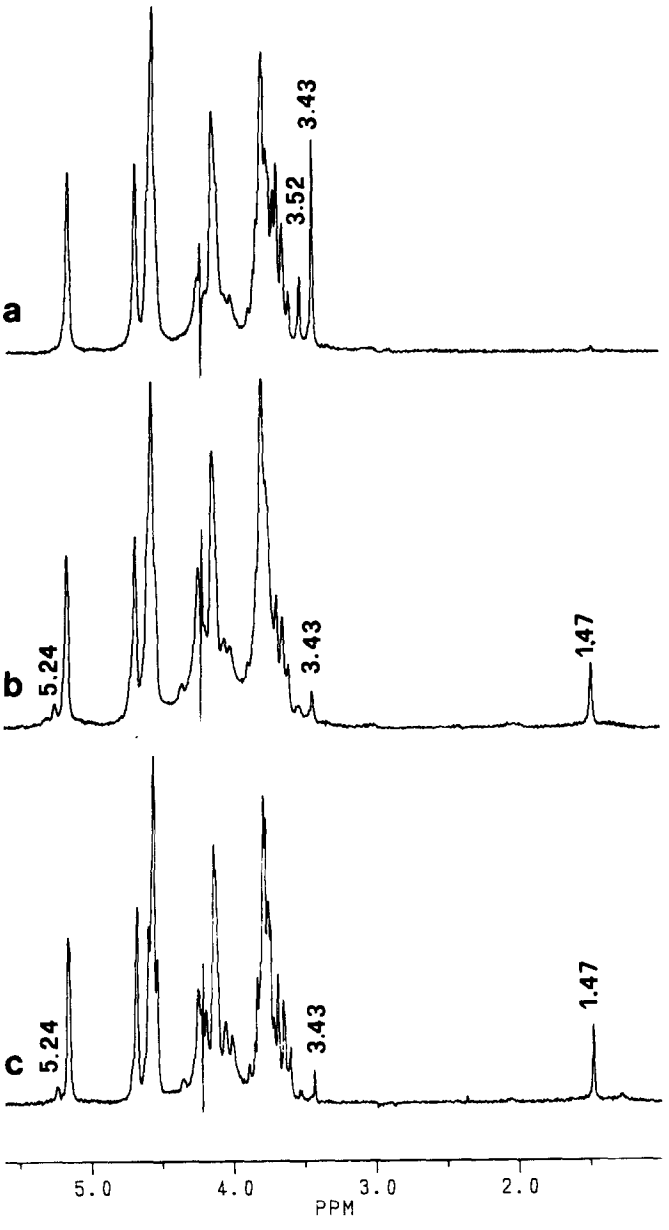

Fig. 1. ^{1}H NMR spectra of the commercial agarose (a) and agar polymers extracted from *Gracilaria dura* without (b) and with (c) alkali pre-treatment. The signals at 3·43 and 3·52 ppm are assigned to the methyl group in 6-*O*-methyl-D-galactose and 2-*O*-methyl-3,6-anhydro-L-galactose, respectively. The signal at 1·47 ppm is assigned to the methyl group of the pyruvate ketal substituent on the D-galactose residue. The low signal at 5·24 ppm is attributed to the anomeric proton of the L-galactopyranose residue adjacent to a pyruvate substituted D-galactose residue. Spectra were acquired at 80 °C using 16K data points. The WEFT technique was applied to suppress the solvent signal.

pointed out that this substitution was significantly higher in the alkali-treated extract than in the native agar (Table 1). On the other hand, no signal attributable to the presence of pyruvate was detected in the ^{1}H NMR spectrum of the commercial agarose (Fig. 1(a)). The low intensity signals appearing at 3·43 and 3·52 ppm in the *G. dura* spectra (Fig. 1(b) and 1(c)) were assigned to the methoxy groups of 6-*O*-methyl-D-galactose and 2-*O*-methyl-3,6-anhydro-L-galactose, respectively (Lahaye *et al.*, 1986). The degree of substitution (DS) estimated from the integral of the signals at 3·43 and 3·52 ppm showed that a reduction in DS was caused by alkali pre-treatment on both galactose residues (Table 1). In contrast to these findings, high intensity methoxy signals in the commercial sample spectrum (Fig. 1(a)) indicated a considerable degree of methylation, mainly at C-6 of the D-galactose residue, as reported in Table 1.

The ^{13}C NMR spectra of all the agar samples investigated showed 12 major signals assigned to the carbons of agarobiose units (Usov *et al.*, 1980), as shown in Fig. 2. The minor signal at 59·1 ppm, typical of *O*-methylated agarobiose, was present in the spectrum of the commercial sample (Fig. 2(a)), but not detectable in the spectra of agar polymers from *G. dura* (Fig. 2(b) and (c)). Furthermore, in the case of the commercial agarose, additional minor signals (Fig. 2(a)) were attributable to agarobiose containing 6-*O*-methyl-D-galactose (G′5, 73·6 ppm and G′6, 71·8 ppm) and 2-*O*-methyl-L-galactose (A″1, 98·7 ppm, A″2, 78·9 ppm and A″3, 78·5 ppm), respectively. In contrast, no detectable signals related to methoxy substituents were present in the ^{13}C NMR spectra of agars extracted from *G. dura*. On the other hand, the presence of 4,6-(1-carboxyethylidene)-β-D-galactose in both the *G. dura* extracts were revealed by a minor signal at 25·7 ppm, assigned to the pyruvate methyl group (Gorin *et al.*, 1982). The remaining low intensity signals (Fig. 2(b) and (c)) were then attributed to the influence of pyruvate ketals on the carbons of the same residue (G°3, 79·5 ppm, G°4, 71·6 ppm, G°5, 66·7 ppm and G°6, 65·3 ppm) (Lahaye, 1986).

Molecular weights

The weight-average molecular weight ($\overline{M}_w$) of the native agar from *G. dura* was remarkably high (393 000) if compared with the commercial sample (176 000), whereas the alkali pre-treatment significantly reduced the molecular weight of the native polymer, as seen in Table 2. The treatment also affected the polydispersity index ($\overline{M}_w/\overline{M}_n$, $\overline{M}_n$ being the number-average molecular weight), which reflects the broadness of the molecular weight distribution curve. In fact, the distribution curve for the alkali-treated sample, giving $\overline{M}_w/\overline{M}_n$ = 3·15, was broader and shifted towards the low molecular weights as compared to the native polysaccharide and the commercial agarose (Fig. 3 and Table 2).

Rheological properties

The native polysaccharide extracted from *G. dura* had a relatively low gel strength if compared with the commercial agarose, as seen in Table 3. On the other

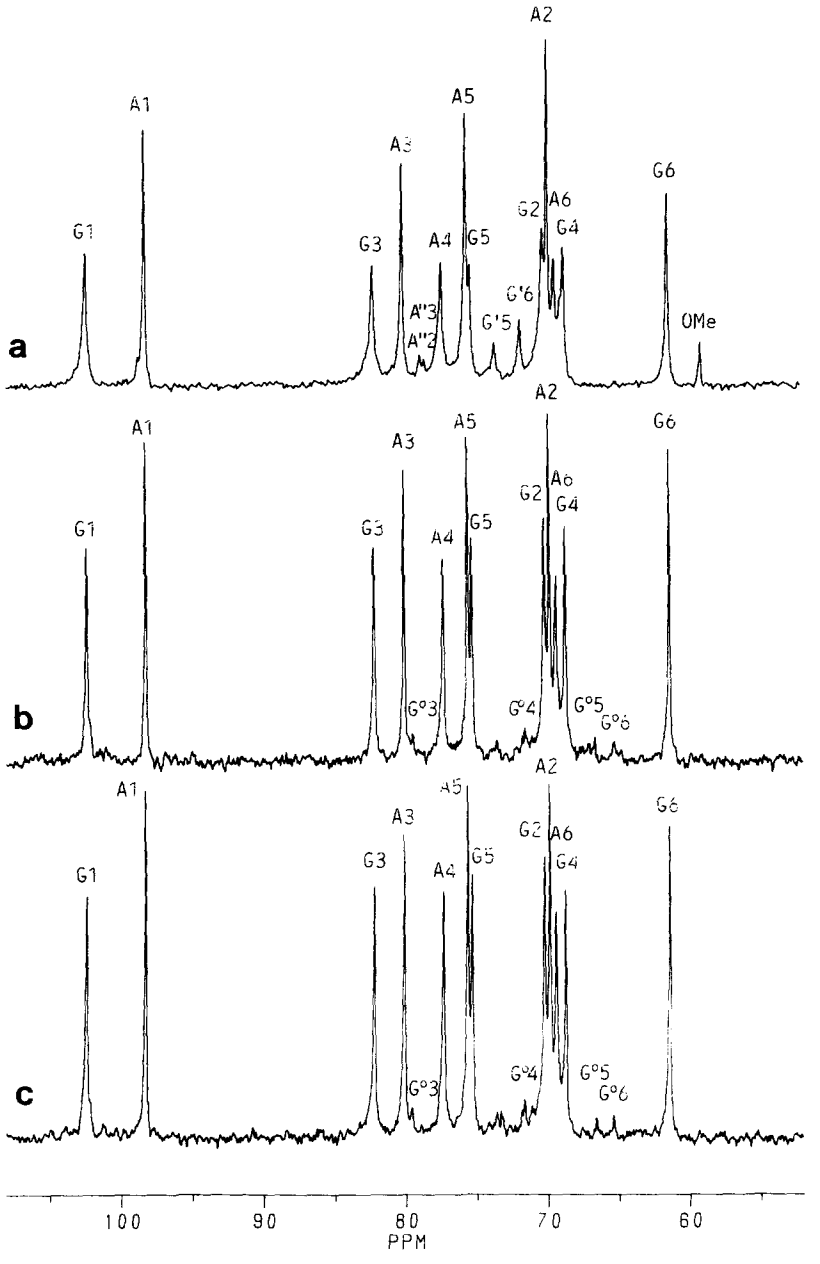

Fig. 2. ^{13}C NMR spectra of the commercial agarose (a) and agar polymers extracted from *Gracilaria dura* without (b) and with (c) alkali pre-treatment. G and A refer to carbons in D-galactose and 3,6-anhydro-L-galactose of agarobiose, respectively. G° corresponds to carbons of the D-galactose residue with the pyruvate ketal substituent; the signal at 25·7 ppm assigned to the carbon of the methyl group is not shown. G′ and A″ refer to carbons in 6-*O*-methyl-D-galactose and 2-*O*-methyl-3,6-anhydro-L-galactose, respectively; OMe refers to the carbon of the methyl group. Spectra were acquired overnight at 80°C using 32K data points.

hand, as expected, the alkaline treatment markedly increased the gel strength. The gelling temperature of the commercial sample was 37°C, whereas the agars extracted from *G. dura* gave an unexpectedly low gelling temperature (31°C). Similarly, the melting temperature of the native agar was lower than that of the commercial sample (Table 3), but a slightly higher value (82°C) was measured for the alkali-treated material.

DISCUSSION

The results presented above refer to agar polymers extracted from winter samples of *Gracilaria dura*, a mediterranean species of Rhodophyta not yet investigated as a potential source of agar. The total yield of agar extracted as native polymer was higher than that of the alkali-treated sample. These findings are in agreement with previous observations (Armisen &

Table 2. Weight-average molecular weight ($\overline{M}_w$) and polydispersity index ($\overline{M}_w/\overline{M}_n$[a]) of the commercial agarose and agar polymers extracted from *Gracilaria dura*

Sample	$\overline{M}_w \times 10^{-3}$	$\overline{M}_w/\overline{M}_n$
Commercial	176 ± 5	2·55
Native	393 ± 10	2·10
Alkali treated	174 ± 5	3·15

[a] $\overline{M}_n$ = number-average molecular weight.

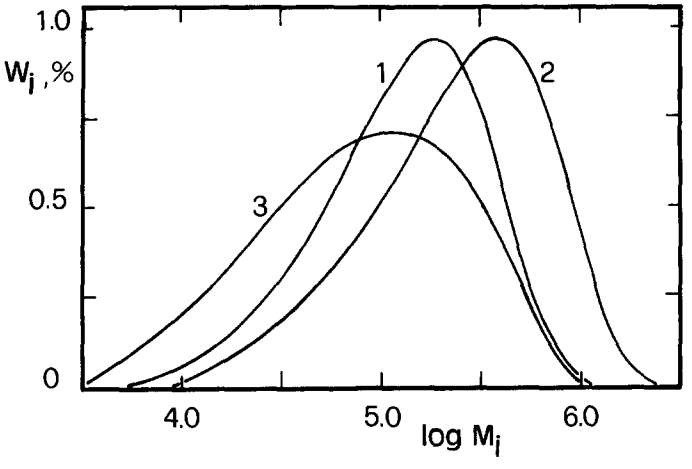

Fig. 3. Differential molecular weight distribution curves from GPC/LALLS experiments of the commercial agarose (1) and agar polymers extracted from *Gracilaria dura* without (2) and with alkali pre-treatment (3). W_i is the weight fraction of polymer of molecular weight M_i.

Galatas, 1987) regarding the yield loss due to polymer degradation caused by alkaline hydrolysis.

NMR spectroscopy of both the native and the alkali-treated extracts from *G. dura* showed the characteristic repeating sequence of the agarose structure (Figs 1 and 2). However, a low methoxyl content and a fairly high amount of pyruvate were found (Table 1). The low degree of methylation could also be deduced from the low gelling temperature (31 °C) for both the agar solutions (Table 3). This parameter is directly related to the methoxyl content (Guiseley, 1970). Such a low gelling point is quite unusual for agars extracted from the genus *Gracilaria*, which usually gel at 40–52 °C (Womer, 1982), and it is the lowest value reported for a 1·5% native agar extracted from this taxon. In fact, Santos & Doty (1983) reported 35–36 °C as the gel setting temperature for the low-methylated agar (1%) extracted from *Gracilaria cylindrica*. Craigie *et al.* (1984) found that a 1% solution of alkali-modified agar from *Gracilaria sjoestedtii* gelled at 33 °C and defined the correspondingly low methoxyl content as exceptional for a *Gracilaria* agar. Whyte & Englar (1980), describing the chemical composition of agars from morphotypes of *Gracilaria*, found a very low methoxyl content in the native agar from the *chorda* type and measured a gelling point of 30·8 °C for a 1% agar solution.

The methoxyl content of an agar of *Gracilaria* origin is mainly attributed to 6-*O*-D-methyl-galactose and 2-*O*-methyl-3,6-anhydro-L-galactose (Ji Minghou *et al.*, 1988). Lahaye *et al.* (1986) reported a variety of degrees of methyl substitution (DS) for different species of the genus *Gracilaria*: for *G. tenuistipitata* a DS with 6-*O*-methyl-galactose in the range 0·13–0·70; for *G. eucheumoides* an unusually high content of 2-*O*-methyl-3,6-anhydro-L-galactose, which ranged from DS 0·7 to 1·0; finally for *G. blodgettii* only traces of 6-*O*-D-galactose and a low DS (0·1–0·2) for 2-*O*-methyl-3,6-anhydro-L-galactose. In the case of agar from *G. dura* we found a different methylation pattern for the 3-linked and 4-linked residues, characterised by exceptionally low DS in the native polymer (0·04 and 0·09) and even lower DS in the alkali-treated agar (0·02 and 0·06). Nevertheless, the reduction of the methoxyl content after the alkaline treatment did not affect at all the gelling temperature of *G. dura* agar, possibly suggesting that below a certain DS value the methoxy groups have practically no effect on the physical texture of the gel.

As far as the pyruvate content is concerned, the polysaccharides from *G. dura* may be considered as having a fairly high pyruvate content. In fact, according to Armisen & Galatas (1987), the quantity of pyruvic acid in agars ranges from 0·2 to 2·5% (w/w). Unexpectedly, in the alkali-treated sample a higher pyruvate content was found (Table 1), which may be attributed to the fact that alkaline conditions preferentially promote the degradation of polymeric regions low in pyruvate content.

Agar polymers obtained from *G. dura* without alkali pre-treatment exhibited a higher molecular weight and a lower polydispersity index than those extracted under alkaline conditions (Table 2, Fig. 3). These data are in

Table 3. Rheological properties of 1·5% (w/v) solutions of the commercial agarose and agar polymers extracted from *Gracilaria dura*

Sample	Gel strength ($g\ cm^{-2}$)	Gelling temperature (°C)	Melting temperature (°C)
Commercial	500	37	87
Native	160	31	80
Alkali treated	390	31	82

agreement with the results previously reported by Rochas & Lahaye (1989), even though the average molecular weights ($\overline{M}_w$) given by these authors for agars extracted from some *Gracilaria* and *Gelidium* species (24 000–124 000) are remarkably lower than that of the native agar from *G. dura* (393 000). In fact, some reduction in molecular weight of the agar chains can be caused by using unsuitable pH and redox extraction conditions, in addition to the present finding that alkali pre-treatment has an important degrading effect on agar polysaccharides. This would suggest that even the molecular weight determined for polysaccharides extracted without alkali pre-treatment should not be considered as the actual average value of the native galactans as they occur in the algal cell wall, but rather as an estimate of its lower bound.

The HPGPC–LALLS-based technique described above is an accurate method for the absolute molecular weight determination of agar polymers and a useful means of correlating gelling properties of agars with their molecular weight distribution. The use of DMSO as the solvent allows uncharged agar fractions to be easily solubilised (Tagawa, 1965), preventing the disorder–order transition during sol–gel interconversion (Arnott *et al.*, 1974). In addition, the use of TBABr depletes the electrostatic interactions due to the charged groups present on the polymer chain.

The gel strength of the agar polysaccharides extracted from *G. dura* was markedly improved by alkali pre-treatment, but it was significantly lower than that of the commercial sample, which had the same molecular weight (Tables 2 and 3). The gel strength increase induced by the formation of 3,6-anhydro-L-galactose and its decrease, ascribed to chain cleavage (Nishinari & Watase, 1983), do not completely account for the discrepancy between the two values. This result may be interpreted in terms of de-esterification and chain breakage, induced by alkali pre-treatment, together with the detrimental effect of the alkali-stable charged groups. In the case of the alkali-treated sample, alkali-stable sulphate esters and pyruvate ketals may increase the agar solubility and thus explain the lower gel strength. This behaviour is also in agreement with the lower energy required to melt the gels obtained from *G. dura* agar polymers (Table 3).

The results reported in this paper demonstrate that the agar synthesised by *G. dura* can be considered of good chemical quality, once its native form is modified by alkali pre-treatment. Moreover, the low total degree of *O*-methylation and the relatively high degree of pyruvate content might suggest specific applications for agars extracted from *G. dura*.

Further research on both the distribution and the role of pyruvate ketal groups may also have interesting implications for chemico-taxonomical studies of the *Gracilaria* genus.

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REFERENCES

Araki, C. & Arai, K. (1956). Studies on the chemical constitution of agar-agar. XVIII. Isolation of a new crystalline disaccharide by enzymatic hydrolysis of agar-agar. *Bull. Chem. Soc. Japan*, **29**, 339–45.

Araki, C. & Hirase, S. (1960). Studies on the chemical constitution of agar-agar. XXI. Re-investigation of methylated agarose of *Gelidium amansii*. *Bull. Chem. Soc. Japan*, **33**, 291–5.

Armisen, R. & Galatas, F. (1987). In *Production and Utilization of Products from Commercial Seaweeds*, ed. D.J. McHugh. FAO fishing tech. paper, pp. 1–57.

Arnott, S., Fulmer, A., Scott, W.E., Dea, I.C.M., Moorhouse, R. & Rees, D.A. (1974). The agarose double helix and its function in agarose gel structure. *J. Mol. Biol.*, **90**, 269–84.

Asare, S.O. (1980). Seasonal changes in sulphate and 3,6-anhydrogalactose content of phycocolloids from two red algae. *Bot. Mar.*, **23**, 595–8.

Craigie, J.S. (1990). In *Biology of the Red Algae*, ed. R.G. Sheath & K.M. Cole. Cambridge University Press, New York, p. 240.

Craigie, J.S. & Leigh, C. (1978). In *Handbook of Phycological Methods. Physiological and Biochemical Methods*, ed. J.A. Hellebust & J.S. Craigie. Cambridge University Press, pp. 109–31.

Craigie, J.S. & Wen, Z.C. (1984). Effect of temperature and tissue age on gel strength and composition of agar from *Gracilaria tikvahiae* (Rhodophyceae). *Can. J. Bot.*, **62**, 1665–70.

Craigie, J.S., Wen, Z.C. & van der Meer J.P. (1984). Interspecific, intraspecific and nutritionally-determined variations in the composition of agars from *Gracilaria* spp. *Bot. Mar.*, **27**, 55–61.

Duckworth, M. & Yaphe, W. (1971). The structure of agar. Part I. Fractionation of a complex mixture of polysaccharides. *Carbohydr. Res.*, **16**, 189–97.

Gorin, P.A.J., Mazurek, M., Duarte, H.S., Iacomini, M. & Duarte, J.H. (1982). Properties of ^{13}C NMR spectra of *O*-(1-carboxyethylidene) derivatives of methyl β-D-galactopyranoside: models for determination of pyruvic acid acetal structures in polysaccharides. *Carbohydr. Res.*, **100**, 1–15.

Guiseley, K.B. (1970). The relationship between methoxyl content and gelling temperature of agarose. *Carbohydr. Res.*, **13**, 247–56.

Gupta, R.K. (1976). Dynamic range problem in Fourier transform NMR. Modified WEFT pulse sequence. *J. Magn. Res.*, **24**, 461–5.

Hirase, S. (1957). Studies on the chemical constitution of

agar-agar. XIX. Pyruvic acid as a constituent of agar-agar (Part 2). Isolation of a pyruvic acid-linking disaccharide derivative from the methanolysis products of agar. *Bull. Chem. Soc. Japan*, **30**, 70–5.

Hoyle, M.D. (1978). Agar studies in two *Gracilaria* species (*G. bursapastoris* (Gmelin) Silva and *G. coronopifolia* J. Ag.) from Hawaii. II. Seasonal aspects. *Bot. Mar.*, **21**, 347–52.

Izumi, K. (1972). Chemical heterogeneity of the agar from *Gracilaria verrucosa*. *J. Biochem.*, **72**, 135–40.

Izumi, K. (1973). Structural analysis of agar-type polysaccharides by NMR spectroscopy. *Biochim. Biophys. Acta*, **320**, 311–17.

Ji Minghou, Lahaye, M. & Yaphe, W. (1985). Structure of agar from *Gracilaria* spp. (Rhodophyta) collected in the People's Republic of China. *Bot. Mar.*, **28**, 521–8.

Ji Minghou, Lahaye, M. & Yaphe, W. (1988). Structural studies on agar fractions extracted sequentially from chinese red seaweeds: *Gracilaria sjoestedtii*, *G. textorii* and *G. salicornia* using ^{13}C-NMR and IR spectroscopy. *Chin. J. Oceanol. Limnol.*, **6**, 87–103.

Karamanos, Y., Ondarza, M., Bellanger, F., Christiaen, D. & Moreau, S. (1989). The linkage of 4-*O*-methyl-L-galactopyranose in the agar polymers from *Gracilaria verrucosa*. *Carbohydr. Res.*, **187**, 93–101.

Kennedy, J.F., Stevenson, D.L., White, C.A., Tolley, M.S. & Bradshow, I.J. (1984). A simple ^{1}H NMR method for the quantitation of *O*-acetate ester and pyruvic acid acetal content of polymeric xanthan gum and related polysaccharides. *Br. Polym. J.*, **16**, 5–10.

Lahaye, M. (1986). Agar from *Gracilaria* spp. PhD thesis, McGill University, Montreal, Canada.

Lahaye, M. & Yaphe, W. (1988). Effects of seasons on the chemical structure and gel strength of *Gracilaria pseudoverrucosa* agar (Gracilariaceae, Rhodophyta). *Carbohydr. Polym.*, **8**, 285–301.

Lahaye, M., Rochas, C. & Yaphe, W. (1986). A new procedure for determining the heterogeneity of agar polymers in the cell walls of *Gracilaria* spp. (Gracilariaceae, Rhodophyta). *Can. J. Bot.*, **64**, 579–85.

Lahaye, M., Revol, J.F., Rochas, C., McLachlan, J. & Yaphe, W. (1988). The chemical structure of *Gracilaria crassissima* (P. *et* H. Crouan in Schramm *et* Mazé) P. *et* H. Crouan in Schramm *et* Mazé and *G. tikvahiae* McLachlan (Gigartinales, Rhodophyta) cell wall polysaccharides. *Bot. Mar.*, **31**, 491–501.

Levy, I. & Friedlander, M. (1990). Strain selection in *Gracilaria* spp. *Bot. Mar.*, **33**, 339–45.

Lewis, J.G., Stanley, N.F. & Guist, G.G. (1988). In *Algae and Human Affairs*, ed. C.A. Lembi & J.R. Waaland. Cambridge University Press, New York, pp. 205–36.

Martinsen, A., Skjåk-Bræk, G., Smidsrød, O., Zanetti, F. & Paoletti, S. (1991). Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates. *Carbohydr. Polym.*, **15**, 171–93.

Nishinari, K. & Watase, M. (1983). Effect of alkali pretreatment on the rheological properties of concentrated agar-agar gels. *Carbohydr. Polym.*, **3**, 39–52.

Painter, T.J. (1983). In *The Polysaccharides*, Vol. 2, ed. G.O. Aspinall. Academic Press, New York, pp. 195–285.

Rees, D.A. (1961). Estimation of the relative amounts of isomeric sulphate esters in some sulphated polysaccharides. *J. Chem. Soc.*, 5168–71.

Renn, D.W. (1984). Agar and agarose: indispensable partners in biotechnology. *I&EC Product Research and Development*, **23**, 17–21.

Rochas, C. & Lahaye, M. (1989). Average molecular weight and molecular weight distribution of agarose and agarose-type polysaccharides. *Carbohydr. Polym.*, **10**, 289–98.

Santos, G.A. & Doty, M.S. (1983). Agarose from *Gracilaria cylindrica*. *Bot. Mar.*, **26**, 31–4.

Skjåk-Bræk, G. & Martinsen, A. (1991). In *Seaweed Resources in Europe: Uses and Potential*, ed. M.D. Guiry & G. Blunden. John Wiley & Sons, pp. 219–56.

Tagawa, S. (1965). Separation of agar-agar by dimethylsulfoxide into agarose and agaropectin. *J. Shimonoseky Univ. Fish.*, **14**, 165–71 (in Japanese).

Tagawa, S. & Kojima, Y. (1972). The alkali-treatment of the mucilage of *Gracilaria verrucosa*. *Proc. Int. Seaweed Symp.*, **7**, 447–50.

Usov, A.I., Yarotsky, S.V. & Shashkov A.S. (1980). ^{13}C-NMR spectroscopy of red algal galactans. *Biopolymers*, **19**, 977–90.

Watase, M. & Nishinari, K. (1983). Rheological properties of agarose gels with different molecular weights. *Rheol. Acta*, **22**, 580–7.

Welti, D. (1977). Carrageenans. Part 12. The 300 MHz proton magnetic resonance spectra of methyl β-D-galactopyranoside, methyl 3,6-anhydro-α-D-galactopyranoside, agarose, kappa-carrageenan and segments of iota-carrageenan and agarose sulphate. *J. Chem. Res. (S)*, 312–13.

Whyte, J.N.C. & Englar, J.R. (1980). Chemical composition and quality of agars in the morphotypes of *Gracilaria* from British Columbia. *Bot. Mar.*, **23**, 277–83.

Whyte, J.N.C., Englar, J.R., Saunders, R.G. & Lindsay, J.C. (1981). Seasonal variations in the biomass, quantity and quality of agar, from the reproductive and the vegetative stages of *Gracilaria* (*verrucosa* type). *Bot. Mar.*, **24**, 493–501.

Womer, M.C. (1982). *The Agarose Monograph*. Marine Colloids Division, FMC Corporation, Rockland, ME, Part 3, pp. 16–22.

Yaphe, W. & Arsenault, G.P. (1965). Improved resorcinol reagent for the determination of fructose and of 3,6-anhydrogalactose in polysaccharides. *Anal. Biochem.*, **13**, 143–8.