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Gelling properties of water-soluble polysaccharides from proliferating marine green seaweeds (*Ulva* spp.)

M. Lahaye[a] & M.A.V. Axelos[b]

[a]*Laboratoire de Biochimie et Technologie des Glucides,* [b]*Laboratoire de Physico-Chimie des Macromolécules, Institut National de la Recherche Agronomique, BP 527, 44026 Nantes Cedex 03, France*

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Water-soluble polysaccharides (12·2% of the algal dry weight) were extracted from marine green seaweed (*Ulva* spp.) which proliferate along the Brittany shores of France. They were composed of 18·4% rhamnose, 4·4% glucose, 1·9% xylose, 0·9% mannose, 0·9% galactose, 15·2% uronic acid, 15·8% sulphate and 23·7% ash based on the extract dry weight. These polysaccharides, formed a weak gel (about 3 Pa) at a concentration of 1·6% (w/v) in deionized water. The elastic modulus increased to about 160 Pa when boric acid (15–33 mM) was added and reached 250 Pa when both boric acid (7 mM) and calcium chloride (7 mM) were present. Adjusting the pH to 7·5 or higher by sodium tetraborate, phosphate or Tris-HCl buffers was detrimental to the gel. These results demonstrate that the poorly exploited biomass of *Ulva* spp. is a source of gelling polysaccharides of potential economical value. Mechanisms for gel formation which unusually involve both boron and calcium ions are proposed and will be studied further.

INTRODUCTION

Marine green seaweeds from the Ulvaceae family (*Ulva* and *Enteromorpha* spp.) represent an important biomass along European shores. Part of this biomass arises from algae proliferation as a consequence of the eutrophication of coastal waters (Briand, 1991). This has a detrimental effect on the environment and local economies (Brittany, Venice lagoon, etc.) and is only partially solved by collecting the plants. Unfortunately, the seaweeds have little value and are used either as compost (Morand *et al.*, 1991) or poultry feed (Briand, 1991) but are often simply dumped. It is also possible that they could be used as a food (sea vegetable) since they are low-calorie and rich in vitamins, trace elements and dietary fibre (Ito & Hori, 1989; Lahaye & Jegou, 1993). The extraction and purification of particular high value compounds, such as gelling polysaccharides, could also stimulate the exploitation of this algal resource. To the knowledge of the authors, there are only two reports in the literature mentioning the gelling ability of *Ulva* water-soluble polysaccharides (McKinnel & Percival, 1962; Haug, 1976) and the latter author indicated the requirement of a basic pH, calcium and boron ions for gelation.

We now report on the gelling characteristics of water-soluble polysaccharides extracted from *Ulva* spp. with respect to pH, buffer type and amount of calcium and boron ions added.

MATERIALS AND METHODS

Algae

Dried (60°C) *Ulva* spp. from 'green tides' was provided by the Centre d'Etudes et de Valorisation des Algues (Pleubian, France).

Extraction

Algal powder (40 g) was suspended in deionized water (500 ml) brought to pH 5·0 by the addition of 1 M HCl; cellulase (Cellucast, Novo Industrie, Bagsvaerd Denmark; 1 ml) was added and the suspension was left at 50°C for 2 h. The final pH was adjusted to 7·0 with 1 M sodium hydroxide and protease (Alcalase, Novo; 1 ml) was added; the suspension was left for 2 h at 50°C and then 30 min in a boiling water-bath. The suspension was centrifuged (2500*g*, 30 min) and the pellet re-extracted in

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deionized water (400 ml) for 30 min in a boiling water-bath. The suspension was centrifuged as above and the two supernatants were pooled, filtered through glass-fibre paper (GFD, Whatman) and a sintered glass filter (porosity 5–15 μm), concentrated and desalted by ultrafiltration through hollow fibres of molecular weight cutoff 10000 (Amicon HP10-20), and precipitated into 4 volumes of 95% ethanol. The precipitate was recovered, washed with 95% ethanol, dehydrated by acetone and diethyl-ether and dried at 40°C *in vacuo* over P_2O_5 (yield 12·2%).

Chemical analysis

Protein content was determined by the micro-Kjeldahl method (N × 6·25). Ash was determined gravimetrically after incineration of samples at 550°C for 16 h followed by 2 h at 900°C. Calcium and boron contents were determined by ICP emission spectroscopy on a Jobin Yvon 38 Plus Séquentiel instrument. Neutral sugars were determined after hydrolysis for 3 h at 120°C in 2 N trifluoroacetic acid (Quemener & Thibault, 1990). Reduction and acetylation of sugars was as described by Harris *et al.* (1984). Monosaccharides were analysed by gas-liquid chromatography on a BP 225 (Scientific Glass Engineering Sarl, Villeneuve-St-Georges, France) fused silica capillary column operating isothermally at 220°C and eluted with H_2. Uronic acids were quantified colorimetrically by the automatized method of Thibault (1979) using glucuronic acid as standard. Sulphate content was measured by HPLC according to the following method. Sample (3–5 mg) was hydrolysed by 2 M HCl (0·5 ml, 2 h, 100°C); after cooling to room temperature, the precipitate formed upon addition of 0·5 ml 95% ethanol was removed by centrifugation in a bench-top centrifuge and a precise volume of the supernatant was evaporated to dryness under a stream of air (50°C). The dry sample was redissolved in deionized water (1 ml) and centrifuged as above before injection on a Nucleosil Anion II column (Macherey-Nagel, Düren, Germany) eluted by 2 mM potassium hydrogen phthalate (pH 5·7, 2 ml min^{-1}). Elution was monitored by conductimetry.

^{13}C-Nuclear magnetic resonance spectra from 5–10% polysaccharide solutions (w/v) in deuterium oxide were recorded at 60°C on a Bruker AM 500 instrument operating at 125·76 MHz. Chemical shifts in parts-per-million were determined from internal dimethylsulfoxide assigned at 39·6 ppm relative to tetramethylsilane.

Rheological experiments

Dynamic rheological measurements were performed using a Carri-med CS-50 controlled-stress rheometer with a temperature-regulated cone–plate device (radius 2 cm, cone–plate angle 4°). An oscillatory movement of a small selected maximum amplitude at a given frequency (ν) is imposed on the upper cone about a vertical axis, which determines a sinusoidal shear stress within the sample. The cone is linked to a stress-controlling device that adjusts the torque necessary to maintain the imposed deformation. In the range of small deformations, the viscoelastic behaviour is linear, complex stress σ^* and strain ε^* are proportional, and their ratio is the complex shear modulus G^* (Ferry, 1980):

$$G^* = \sigma^*/\varepsilon^* = G' + iG'' = |G^*|(\cos\phi + i\sin\phi)$$

The real part of the complex modulus, G' , is the storage modulus, and it represents the elastic response of the system to an applied stress (in-phase). In the gels the predominant factors are the degree of crosslinking of the sample, and the lifetime of the crosslinks. The imaginary part G'' is the loss modulus, corresponding to the energy dissipated in the system through viscous flow (out of phase). ϕ represents the loss angle. In a viscoelastic solid (referred to as a gel), a frequency-independent storage modulus exists over a broad frequency range in the limits of the experimental conditions, thus an equilibrium elastic modulus G'_e may be defined as the limit of $G'(\nu)$ as $\nu \rightarrow 0$. The polysaccharide concentrations in buffer solutions and salt solutions were 1·6 to 1·8% (w/v) and experiments were carried out at 20°C. Calcium and boron ions were added just before pouring the polysaccharide solution onto the Carri-med plate. Paraffin oil was layered onto the plunger cone and sample to prevent evaporation of the sample. The kinetics of gel formation was monitored by measuring the complex modulus at 0·159 Hz (1 rad s^{-1}) with a low deformation of 0·03, for about 24 h, or until a plateau was reached. Experimental conditions were checked so as not to perturb the system. Mechanical spectra were recorded in a constant strain mode with the same low deformation of 0·03 maintained over the frequency range between 5×10^{-3} and 10 Hz.

RESULTS

Hot water extraction of dry *Ulva* powder after cellulase and protease treatments allowed for the recovery of 12·2% polysaccharides. The extract is composed of 41·7% sugars, 15·8% sulphate, 4·3% protein and 23·7% ash on a dry weight basis. Calcium and boron amounted to 1·98% and 0·073% on the dry weight basis of the polysaccharides. The sugar composition shown in Table 1

Table 1. Chemical composition of the water-soluble polysaccharide from proliferating *Ulva* spp. (ulvan)

Uronic acids	Rha	Xyl	Man	Gal	Glc	SO_4
			mol %			
19·3	28·2	3·2	1·3	1·3	5·9	40·8

is in agreement with that of water-soluble polysaccharides from other members of the Ulvaceae family (Percival & McDowell, 1967; Abdel-Fattah & Edrees, 1972; Magdel-Din Hussein, 1977; Yamamoto, 1980). Although glucose could have arisen from starch, the typical chemical shifts for such polymers were absent from the ^{13}C NMR spectrum of the polysaccharides (data not shown). For the sake of simplicity, these sulphated rhamnoglycuronans will be referred to as ulvan and work is now in progress to determine their fine chemical structure.

The influence of pH on gel formation of ulvan was investigated first. The polysaccharides were dissolved in phosphate buffer at different pH and sodium tetraborate ($Na_2B_4O_7.10H_2O$) was added just before pipetting the solution onto the rheometer plate. The evolution of the elastic modulus (*G'*) with time obtained at different pH (Fig. 1) demonstrated that gel formation was time-dependent and was optimum for a narrow pH range centred at about 7·5. A constant value for *G'* was always reached within 1 h.

Since some turbidity was observed in the ulvan solution prior to the addition of sodium tetraborate and because interactions between the minor amount of calcium in ulvan and phosphate ions from the buffer were suspected, polysaccharide solutions were made in Tris-HCl at pH 7·5 which was the buffer used by Haug (1976). In this buffer no turbidity was observed but the gelation kinetics markedly differed and a higher elastic modulus was obtained, probably reflecting the slightly higher sodium tetraborate concentration used (7 mM in Tris-HCl instead of 4·5 mM in phosphate buffer; Fig. 2). Because Haug (1976) described ulvan gelation as dependent on both calcium and boron ions, $CaCl_2$ was added to the polysaccharide solution before addition of sodium tetraborate. The elastic modulus obtained was much lower than without calcium (Fig. 2) even though a slightly higher ulvan concentration was used (1·7% instead of 1·6% w/v without calcium).

Sodium tetraborate, boric acid and a mixture of both forms of boron at pH 7·5 were tested on ulvan gelation. The results indicated that $Na_2B_4O_7$ (7 mM) did not induce gel formation even when calcium was added (data not shown). However, $Na_2B_4O_7/H_3BO_3$ (7 mM) and H_3BO_3 (7 mM) alone induced gel formation and gelation was faster with boric acid (Fig. 2). The pH of the ulvan in deionized water was 7·5; when sodium tetraborate (7 mM) was added, it rose to 9·0; but when H_3BO_3 (7 mM) was added it dropped slightly to 7·4. Thus, buffering the system at a slightly basic pH as reported for the polysaccharides extracted by Haug (1976) was not required for gelation and boric acid was

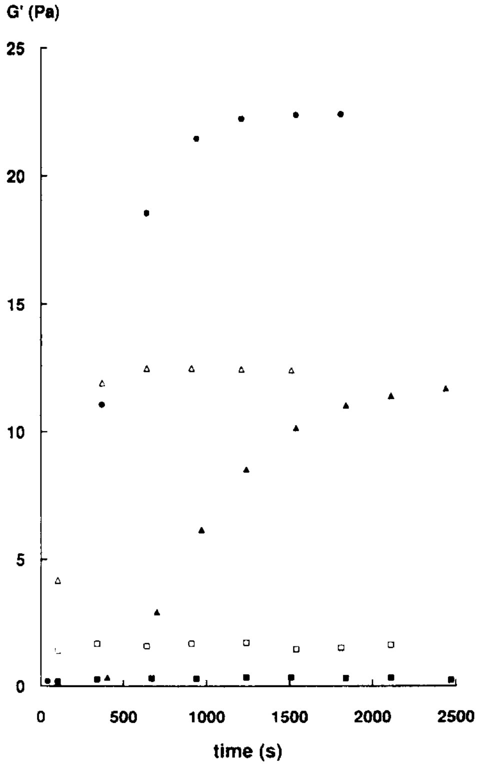

Fig. 1. Effect of pH on the kinetics of the elastic modulus (*G'*), measured at $v = 0{\cdot}159$ Hz, of 1·8% (w/v) ulvan in 0·1 M phosphate buffer containing 4·5 mM sodium tetraborate ■ pH 6·0; □ pH 6·5; △ pH 7·0;. ● pH 7·5; ▲ pH 8·0.

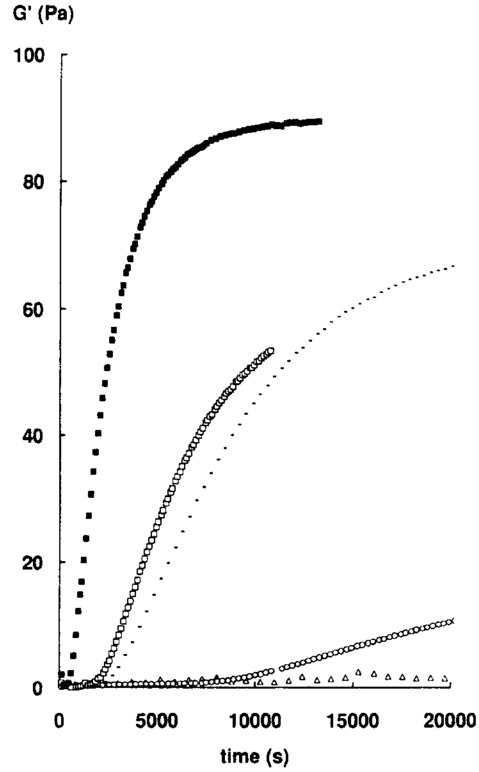

Fig. 2. Effect of different boron species and buffers on the kinetics of the elastic modulus (*G'*), measured at $v = 0{\cdot}159$ Hz of ulvan. Ulvan concentration was: (—) 1·7% (w/v) in 0·1 M Tris-HCl pH 7·5 containing 7 mM sodium tetraborate, (○) 1·6% in 0·1 M Tris-HCl pH 7·5 containing 7 mM sodium tetraborate and 7 mM $CaCl_2$, (△) 1·6% in deionized water, (■) 1·6% in 7 mM boric acid, (□) 1·6% in 7 mM boric acid/sodium tetraborate pH 7·5.

adequate for the gel formation. In deionized water without any added ion, gelation was achieved ($G' > G''$) although G' was very low (about 3 Pa; Fig. 2). The low amount of calcium and boron already present with ulvan (0·79 mM Ca and 10·8 μM B, respectively for a 1·6% ulvan solution) may have been sufficient to promote gelation.

The effects of the concentration of boric acid and calcium on G' were investigated further (Fig. 3). The modulus of elasticity rose rapidly with increasing concentration of boric acid, reached a maximum of about 160 Pa for concentrations between 15 and 33 mM of H_3BO_3 and then dropped markedly at higher concentrations of acid (about 19 Pa at 330 mM; Fig. 3). When calcium was added to ulvan solutions containing 7 mM boric acid, there was a sharp increase in G' with increasing Ca concentration (maximum of about 250 Pa for 7 mM $CaCl_2$) followed by a gradual decrease with additional calcium to reach 160 Pa at about 330 mM $CaCl_2$ (Fig. 3). With this system, the addition of calcium improved the elastic modulus in contrast to the results obtained with Tris-HCl.

In all cases, the constant value for G' and the large G'/G'' ratio observed on the mechanical spectra in the frequency range explored (Fig. 4) indicated that these systems were viscoelastic solids referred to as true gels.

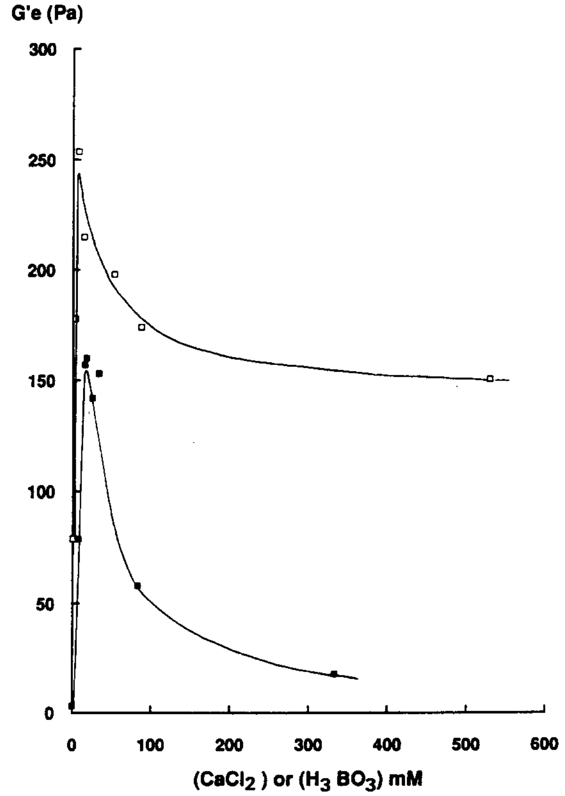

Fig. 3. Effect of the concentration of (■) boric acid alone and (□) calcium chloride with 7 mM boric acid on the equilibrium elastic modulus (G'e) of 1·6% (w/v) ulvan in deionized-water (lines are just guides for the eyes).

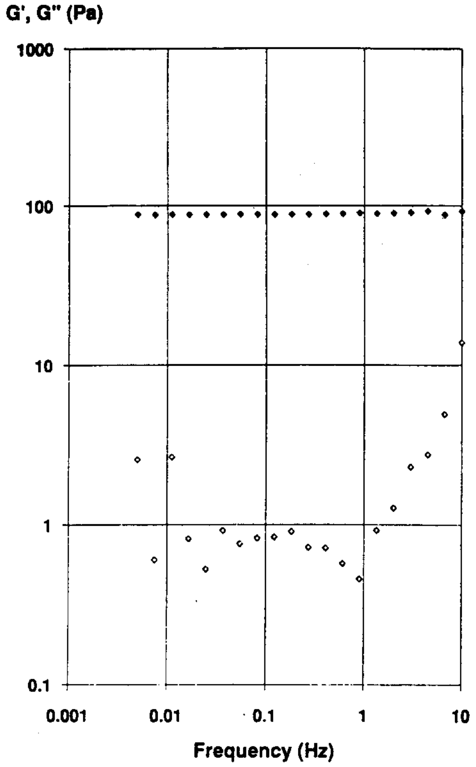

Fig. 4. Mechanical spectrum of ulvan gel (1·6% w/v in 7 mM boric acid); (◆) G' and (◇) G''.

DISCUSSION

Some red and brown macrophytes are industrially exploited for the production of gelling and thickening polysaccharides (agar, carrageenans, alginates; McHugh, 1987) but no green seaweed is used for this purpose. According to Haug (1976), *Ulva lactuca* water-soluble polysaccharides gel at concentrations $\geq 1\%$ (w/v) in seawater or in solutions at pH $\geq 7{\cdot}5$ containing 0·4 mM boric acid and 12·6 mM calcium chloride. Water-soluble polysaccharides extracted from proliferating *Ulva* spp. formed a very weak gel in deionized water but the elastic modulus increased markedly in the presence of boron. In contrast to the results of Haug (1976), there was no absolute requirement for calcium for the gel formation probably because the small amount of this cation already associated with ulvan was sufficient for gel formation. Optimum gel characteristics were found with 15–33 mM boron as boric acid and with about 7 mM calcium under the conditions used. These values cannot be compared to those of Haug (1976) who did not study the effect of variable boron and calcium concentrations on the gelling properties of *U. lactuca* water-soluble polysaccharides. In contrast to Haug's report, buffering the system at pH $\geq 7{\cdot}5$ was detrimental to the gel. This different behaviour may be indicative of structural variations between ulvan from proliferating *Ulva* and *U. lactuca*. Furthermore, buffers such as Tris-HCl and phosphate affected the gel characteristics and the best gels were obtained in the absence of ions other than boric acid.

Polysaccharide– and sugar–boron interactions involve contiguous *cis*-hydroxyls and occur at a basic pH where the borate species is favoured (Pezron *et al.*, 1988*a*; Noble & Taravel, 1987, 1988; Munoz & Lamande, 1991). Such gelling systems have been particularly well described with galactomannan and borax (Pezron *et al.*, 1988*b*, Noble & Taravel, 1990; Noble *et al.*, 1990). For *U. lactuca* ulvan, Haug (1976) proposed associations between borate and free hydroxyls of the polysaccharides and the chelation of calcium by hydroxyls from borate (**1**, **2**).

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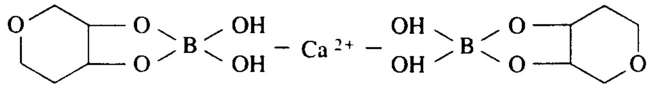

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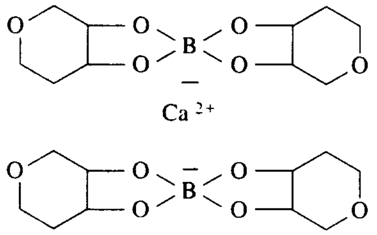
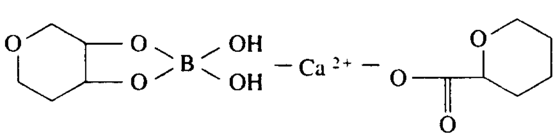


This model also implies the presence of free *cis*-hydroxyls on the different polysaccharidic chains and according to literature and preliminary chemical analysis (Percival & McDowell, 1967, Lahaye unpublished results) the only major sugar presenting such characteristics in ulvan is the 4-linked rhamnose since the uronide residues are 3- and/or 4-linked glucuronic acids. This model requires that O-2 of rhamnose is not substituted by ester sulphate although it has been described as a major site of sulphation in *U. lactuca* water-soluble polysaccharides (Percival & McDowell, 1967).

Considering the molar ratio of sulphate, neutral sugars and uronic acids in ulvan form proliferating *Ulva* spp (Table 1), there must be some rhamnose substituted by several sulphate groups and/or sulphated uronic acids in order to have rhamnose with hydroxyls available for the ionic complex formation. Furthermore, from the presence of acidic moieties on ulvan, other interactions, such as 'ionic-bridges' between the carboxylic group of uronic acids and/or sulphate with borate through divalent cations (**3**, **4**) are also possible in the gel formation.

3



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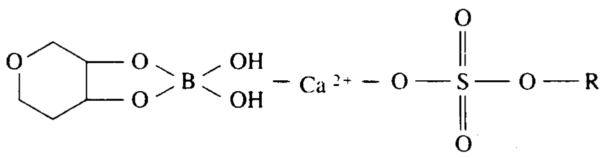

In addition, because gels of ulvan from proliferating algae are not formed at high pH, alkaline-sensitive hydrophobic interactions may also be involved in the gelling mechanism.

The above results demonstrate that the under-used *Ulva* spp. can be source of gelling water-soluble polysaccharides and thus, the biomass generated by these proliferating green algae may be more profitably used. Preliminary experiments on water-soluble polysaccharides from *Entermorpha* spp. which belongs to the ulvaceae family, also formed gels under similar conditions. The precise chemistry and the peculiar mechanism of gelation of these polysaccharides is currently under investigation.

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