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PURIFICATION AND CHEMICAL AND PHYSICAL CHARACTERISATION OF AN ANTITUMOUR POLYSACCHARIDE FROM THE BROWN SEAWEED *Sargassum fulvellum*

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ABSTRACT

A polysaccharide isolated from a hot-water extract of *Sargassum fulvellum* and purified by gel-filtration chromatography on Sepharose 4B inhibited the growth of subcutaneous Sarcoma-180 in mice. The purified, active substance appeared to be sodium alginate having a mol. wt. of 33,400 and a molar ratio of mannuronic acid to guluronic acid of 2.78.

INTRODUCTION

We have reported[1,2] that the hot-water extract of the brown seaweed *Sargassum fulvellum* inhibited the growth of the subcutaneous Sarcoma-180 tumour in mice, and that the active fraction (A), isolated by fractional precipitation with ethanol, was a polysaccharide fraction containing mainly uronic acids together with small proportions of neutral sugars, proteins or peptides, and sulfate groups. Furthermore, electrophoresis indicated the fraction be almost pure[2].

We now report on the purification of fraction A and on the chemical and physical characterisation of the purified substance.

EXPERIMENTAL

General. — Ethanol, 1-butanol, phenol, and acetic acid were distilled before use. D-Mannuronic and L-guluronic acids were prepared[3] from purified samples of commercial alginate (Nakarai Chemicals Ltd.).

T.l.c. was performed on silica gel (Replate 50, 0.2-mm layers, Yamato Scientific Co. Ltd.) after impregnation[4] of the plates with phosphates: *A*, impregnation with 0.5M NaH_2PO_4, 0.1M lactic acid–2-propanol–acetone (1:2:2)[5]; *B*, impregnation with 0.3M NaH_2PO_4, 1-butanol–ethanol–0.1M phosphoric acid (1:10:5)[4]. Detection was effected with diphenylamine–aniline–phosphoric acid[6]. G.l.c. was carried out with a Hitachi gas chromatograph Model 063 equipped with flame-ionisation detector; and *1* (for trimethylsilylated neutral sugars), a glass column (3 mm

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× 2 m) of 3% of SE-30 on Chromosorb W[7] (60–80 mesh) at 150°; *2* (for trimethylsilylated aldono-1,4-lactones of uronic acids), a glass column (3 mm × 1 m) of 10% of poly(neopentylglycol sebacate) on Chromosorb W[8] (100–200 mesh) at 170°; and *3* (for trifluoroacetylated alditols), a glass column (3 mm × 2 m) of 2% of XF-1105 on Gaschrom P[9] (60–80 mesh) at 130°. Electrophoresis (200 V, 15–20 min) was carried out at 20–25° on a cellulose acetate paper Separax (6 × 11 cm, Fuji Film Co. Ltd.) in *A*, 0.1M pyridine–acetic acid buffer (pH 3.5); and *B*, 0.05M borate buffer (pH 9.3); followed by staining with 0.5% of Toluidine Blue in 3% acetic acid or the periodic acid–Schiff reagent[10] as appropriate.

Elemental analyses were performed with a Shimadzu Analyzer Model 240. Ash contents were determined by heating polysaccharide samples to constant weight at 800°. The components of the ash were analysed with a Jarrel Atomic Absorption Spectrophotometer, Model AA-IMK II. Neutral sugars were determined by the anthrone–sulfuric acid method[11], using D-galactose as the standard. Uronic acid was determined by the method of Bitter and Muir[12], using D-mannuronic and L-guluronic acids as standards.

Materials. — The brown seaweed *Sargassum fulvellum* was collected in Japanese waters during the first two weeks of December 1975. The freshly collected seaweed fronds were washed with running water to remove foreign substances and then with deionised water, air-dried, and milled.

Isolation and purification of the antitumour polysaccharide. — An almost pure polysaccharide (fraction A) was prepared from the seaweed fronds of *Sargassum fulvellum* as reported previously[1,2]. A solution of fraction A (270 mg) in 0.2M NaCl (40 mL) was applied to a column (5 × 93 cm) of Sepharose 4B, and eluted with 0.2M NaCl at 36 mL/h. Fractions (15 mL) were monitored on the basis of the phenol–sulfuric acid reaction[13] for carbohydrates and absorption at 260 nm. Fraction A gave three sub-fractions (AI–AIII) (Fig. 1a), of which AII contained most of the polysaccharide material and had little u.v. absorption. Thus, fractions 46–86 were combined, dialysed against distilled water, and lyophilised, to give AII. Likewise, fractions 31–36 and 97–112 gave AI and AIII, respectively. The yields of AI–AIII were 2.5, 73.9, and 2.8%, respectively, of A. Electrophoresis (buffer *A*) showed that AI and AIII were mixtures of two Toluidine Blue-stainable components, of which the more intense staining remained at the origin and the other (very faint staining) migrated towards the anode. On the other hand, AII contained largely one Toluidine Blue-stainable component, which migrated towards the anode, and a trace of stainable material at the origin. Rechromatography of AII on Sepharose 4B was carried out in the same manner as described above (Fig. 1b). Fractions 48–92 gave B (65.2% of A) (Found: C, 35.16; H, 4.47; ash, 16.1 ±1.1%). Fraction B contained no N, S, or P, and the ash contained mainly Na with traces of K, Mg, and Ca.

In electrophoresis (buffers *A* and *B*), fraction B migrated towards the anode as a single band, which was stained with Toluidine Blue and gave the periodate–Schiff reaction.

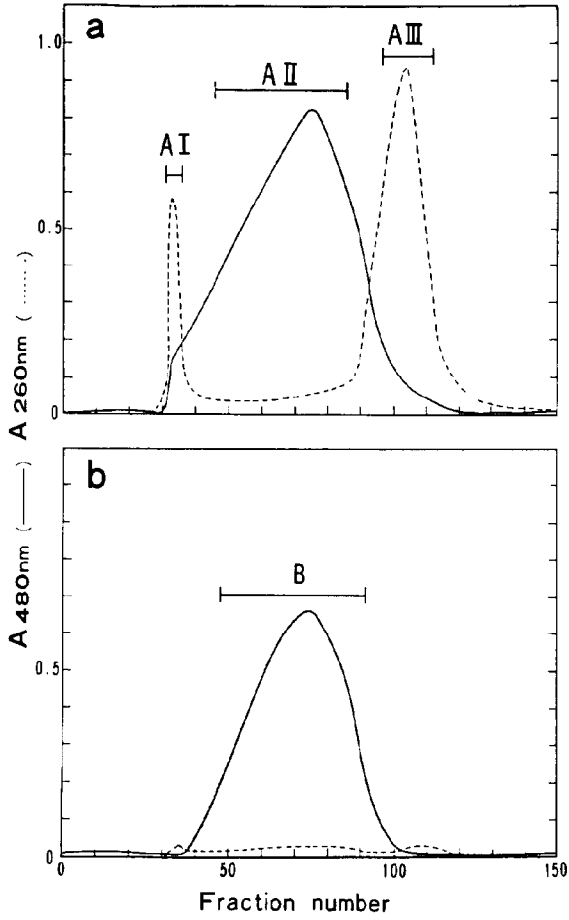


Fig. 1. Chromatography of fractions A (a) and AII (b) on a column (5 × 93 cm) of Sepharose 4B; elution with 0.2M NaCl; 15-mL fractions monitored by the phenol–sulfuric acid reaction and absorbance at 260 nm.

Ion-exchange chromatography[14] *of fraction B.* — A solution of B (154 mg) in water (22 mL) was applied to a column (1.9 × 43 cm) of Whatman DE-23 (Cl^- form) which was eluted with water (360 mL) and then with a linear gradient of 0→0.4M NaCl containing 0.01M HCl, at 3 mL/min. The total volume of the NaCl eluates was 2 L. Fractions (18 mL) were monitored on the basis of the phenol–sulfuric acid reaction and absorbance at 260 nm. Only one carbohydrate fraction was eluted with the NaCl gradient (Fig. 2). Fractions 80–102 gave C (88 mg).

Assay of antitumour activity. — The activity of fractions of AI, AIII, and B was assayed by the following procedure. Seven-day-old Sarcoma-180 ascites cells (1×10^7 cells) were implanted subcutaneously into the back of albino male mice (ddy strain, 20–25 g). A solution of each fraction in normal saline solution was injected

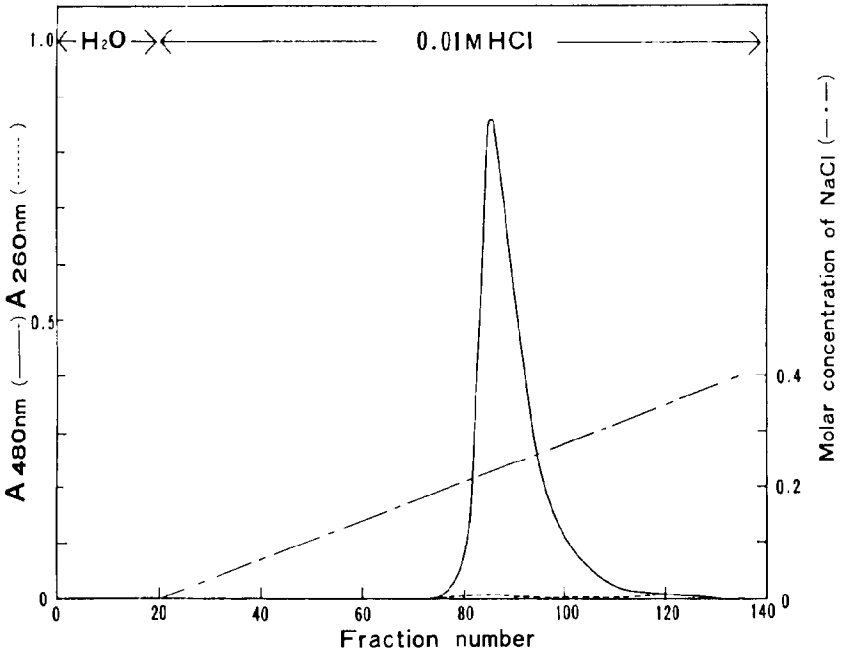


Fig. 2. Chromatography of fraction B (Fig. 1b) on a column (1.9 × 43 cm) of DE-23 (Cl^- form); elution first with water, and then with a linear gradient 0→0.4M NaCl containing 0.01M HCl; 18-mL fractions monitored by the phenol–sulfuric acid reaction and absorbance at 260 nm.

intraperitoneally (10–50 mg/kg daily for 10 days, starting 24 h after tumour implantation). After 5 weeks, the mice were sacrificed, and the tumours were extirpated and weighed. The inhibition ratio (%) is given by [(C − T)/C] × 100, where C is the average tumour-weight of the control group, and T that of the treated group.

Characterisation of fraction B. — (*a*) Fraction B (50 mg) was hydrolysed with sulfuric acid[3], the hydrolysate was neutralised ($CaCO_3$) and centrifuged, and the precipitate was washed thrice with water. The combined supernatant solution and washings were adjusted to pH 8.0 with M NaOH and kept thereat for 30 min, in order to convert lactones into acids. The solution was then added to a column (2.2 × 20 cm) of Dowex 1-X8 (AcO^-) resin (200–400 mesh) and eluted with a linear gradient of 0.5→2.0M acetic acid at 1 mL/min. Fractions (10 mL) were analysed by the phenol–sulfuric acid reaction for carbohydrates. Two carbohydrate fractions were eluted; the peak of the first was at 1.52M acid and that of the second at 1.82M acid. The two fractions (1.4–1.7 and 1.8–2M acid) were concentrated to dryness *in vacuo*. T.l.c. (solvent *B*) revealed guluronic acid and its lactone (R_{GlcA} 0.89 and 1.73, respectively) in the first fraction, and mannuronic acid and its lactone (R_{GlcA} 1.15 and 1.55, respectively) in the second. These results were confirmed by g.l.c. (column 2) of the trimethylsilylated aldono-1,4-lactones (first fraction, R_{GlcA} 0.65; second fraction, R_{GlcA} 1.31). The first fraction was identified as L-gulurono-6,3-lac-

tone, m.p. 141–143°; lit.[15] m.p. 141–142°. The second fraction was characterised, after derivatisation, as D-mannurono-6,3-lactone 2,4-dinitrophenylhydrazone, m.p. 205–208°; lit.[16] m.p. 203–210°.

(*b*) Fraction B was esterified and reduced by the method of Hirst *et al.*[17]. The reduced sample (3.5 mg) was hydrolysed (2M HCl,100°, 6 h), and the products were reduced conventionally with aqueous 1% $NaBH_4$, trifluoroacetylated, and analysed by g.l.c. (column *3*), which revealed derivatives of mannitol ($R_{Xylitol}$ 1.44) and glucitol ($R_{Xylitol}$ 2.08) as the main components together with traces of unknown substances ($R_{Xylitol}$ 0.88 and 2.41).

(*c*) Fraction B was hydrolysed and chromatographed in the same manner as described in (*a*), and the content of each uronic acid was then determined by the method of Bitter and Muir[12]. The contents of mannuronic and guluronic acids were determined as 53.7 ±0.6% and 12.8 ±0.4%, respectively. The uronic acid content was determined before and after hydrolysis of B (phenol–sulfuric acid reaction). The extent of hydrolysis was evaluated as 78.1%.

The M/G ratio (molar ratio of mannuronic acid to guluronic acid) was also determined from the content of each uronic acid by correcting its content according to the method of Haug and Larsen[3]. The ratio was found to be 2.78 ±0.08.

(*d*) Fraction B (31 mg) was hydrolysed (2M HCl, 100°, 6 h) in a sealed tube. The HCl was then completely evaporated *in vacuo*, the residue was diluted with water, and insoluble substances were removed by centrifugation. The supernatant solution was adjusted to pH 8.0 with M NaOH, kept thereat for 30 min in order to convert lactones into acids[3], applied to a column (1.2 × 20 cm) of Dowex 1-X8 (AcO^-) resin (200–400 mesh), and eluted with water (200 mL). The eluate was concentrated to dryness *in vacuo*. T.l.c. (solvent *A*) of the hydrolysate and g.l.c. (column *1*) of the trimethylsilylated products of hydrolysis revealed galactose [t.l.c., R_{Glc} 0.74; g.l.c., $R_{Mannitol}$ 0.70 (major peak) and 0.82 (small peak)]. The galactose content (determined by the anthrone–sulfuric acid method) was 0.23 ±0.01%. Likewise, hydrolysis of fraction C gave galactose, and the content was determined to be 0.22 ±0.03%.

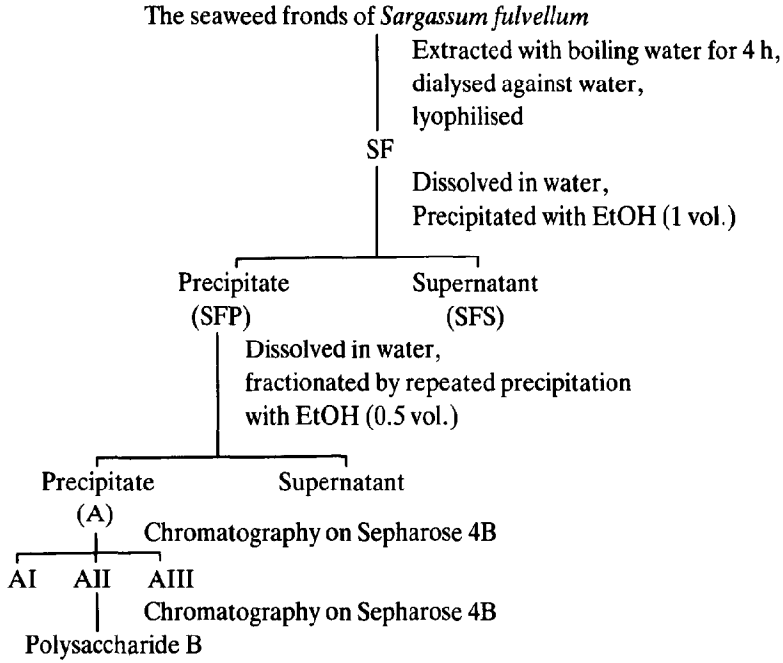
Characterisation of B. — The sedimentation of B (2–10 mg/mL) was carried out in 66mM phosphate buffer (pH 7.0) containing 0.1M NaCl with a Hitachi 282 analytical ultracentrifuge at 20.3 ±0.3° and a rotor speed of 60,000 r.p.m. The sedimentation constant ($S^0_{20,w}$) was determined by extrapolating the $S_{20,w}$ values to zero concentration. Viscosity measurements of B (2.0–6.0 mg/mL) were performed in the above buffer solution with an Ostwald viscometer (flow time, 155 s for water) at 25 ±0.02°. The intrinsic viscosity [η] was determined by extrapolating the observed viscosities to zero concentration. The degree of polymerisation (d.p.) was calculated from the intrinsic viscosity by using the formula of Donnan and Rose[18], and the molecular weight was estimated from the d.p. by using the value of 216 as the equivalent weight of B. Optical rotations were measured for aqueous solutions of B (0.110–0.439 g/100 mL) with a JASCO DIP-4 polarimeter at 22° and at 589 nm. The i.r. spectrum of B (1–2 mg) was recorded for a KBr pellet with a Hitachi

EPI-G2 spectrophotometer. The partial specific volume was determined gravimetrically at 20 ±0.02°.

Detection of bacterial endotoxins (lipopolysaccharides, LPS). — LPS were detected by the gelation test with *Limulus* lysate, using "Limulus Single Test Wako" (Wako Pure Chemical Industries, Ltd., Japan). The *Limulus* test was applied to B before and after treatment[19], in sequence, with aqueous periodate, bromine, and borohydride.

RESULTS AND DISCUSSION

Chromatography of fraction A, obtained[2] as shown in Scheme 1, on Sepharose 4B gave one major (AII) and two minor polysaccharide fractions (AI and AIII) (Fig. 1a). Electrophoresis showed that AII was a mixture of at least two components, but further chromatography on Sepharose 4B gave the major polysaccharide component (B) (Fig. 1b), the homogeneity of which was shown by electrophoresis, ultracentrifugation, and anion-exchange chromatography (see Experimental).



Scheme 1. Isolation and purification procedures for polysaccharide B from *Sargassum fulvellum*.

Fraction B was more active against the Sarcoma-180 tumour in mice at 10 mg/kg than fractions AI and AIII (Table I) and showed substantial activity at 50 mg/kg. The activity of AI and AIII could not be tested at >10 mg/kg because of the low solubility in normal saline. In the previous study[2], electrophoresis (buffer *A*) of fraction SFP (see Scheme 1), and the fractions obtained therefrom by ethanol precipitation, including fraction A, revealed at least two components, one of which remained at the origin while the other(s) migrated towards the anode. Moreover, as the proportion of the origin component decreased, the antitumour activity increased, suggesting that the activity resided in the component(s) which migrated towards the anode. Electrophoresis also showed that both AI and AIII contained a non-migrating component and it is concluded that B is the antitumour-active component of A.

T.l.c. of a hydrolysate of B indicated its uronic acid constituents to be mannuronic and guluronic acids. The latter was characterised as the 6,3-lactone and the former as the 6,3-lactone 2,4-dinitrophenylhydrazone. The yields of mannuronic and guluronic acids were 53.7% and 12.8% of B, respectively. The combined yield (66.5%) was substantially lower than the uronic acid content assayed before hydrolysis (85.1%). The ratio of mannuronic and guluronic acids in B was 2.78.

Fraction B also contained 0.23% of galactose. A similar (0.22%) content of galactose was found in fraction C, obtained by anion-exchange chromatography of B on Whatman DE-23, suggesting that B was not contaminated with neutral galactose-containing material. Fraction B contained no N, S, or P, and the contents of uronic acid, neutral sugar, and ash were 85.1, 0.23, and 16.1%, respectively. Fraction B had i.r. absorption at 890 cm^{-1}, suggesting the presence of β linkages. Moreover, since the major constituent of B was D-mannuronic acid, the $[\alpha]_D^{22}$ value of $-124°$ (water) also suggested the presence of β linkages. The partial specific volume, 0.45 ±0.01 mL/g, was similar to that of sodium alginate[20]. The sedimentation constant $S^0_{20,w}$ was 1.90 ±0.02 S, the intrinsic viscosity $[\eta]$ was 2.67 ±0.16 (100 mL/

TABLE I

ANTITUMOUR ACTIVITY[a] OF FRACTIONS OF A POLYSACCHARIDE (A) ISOLATED FROM A HOT-WATER EXTRACT OF *Sargassum fulvellum*

Fraction	*Dose (mg/kg)*	*Av. tumour wt. (g)*	*Inhibition ratio (%)*	*Complete regression*
B	50 × 10	0.30	84.1	3/9
Control	—	1.89	—	0/17
B	25 × 10	1.21	55.8	0/7
Control	—	2.74	—	0/17
B	10 × 10	1.18	50.0	0/8
AI	10 × 10	1.80	23.7	0/7
AIII	10 × 10	2.26	4.2	0/8
Control	—	2.36	—	0/12

[a]See Experimental for details of the assay.

g), and the d.p.. calculated[18] from $[\eta]$, was 155, corresponding to a molecular weight of 33,400.

Alginic acids, which occur in a wide variety of brown seaweeds[21], vary in the proportions of D-mannuronic and L-guluronic acid residues, and their sodium salts have $[\alpha]_D$ values in the range -120 to $-150°$. Also, the D-mannuronic acid residues are (1→4)-β-linked. Thus, the chemical and physical properties detailed above suggest that fraction B may be a sodium alginate.

Belkin *et al.*[22] have reported on the antitumour activity of alginates, but their polysaccharide was not fully characterised. It is possible that the activity of fraction B against Sarcoma-180 in mice may be due to contaminants which were not detected by ultracentrifugation and electrophoresis. Peptides[23], nucleosides[24], and bacterial lipopolysaccharides[25] (LPS) show marked activity against Sarcoma-180. Fraction B contained no nitrogen, thereby ruling out contamination by peptides, nucleosides, or other nitrogen-containing substances. The presence of bacteria during the isolation of fraction B from *S. fulvellum* could have resulted in contamination by LPS. Application of a *Limulus* test to fraction B suggested the presence of 0.18% of LPS. Suzuki *et al.*[26] investigated the effect of modified polysaccharides on the gelation of *Limulus* lysate and concluded that gelation of the lysate depended on the modified polysaccharide having some anionic character and also on the molecular weight. Similar results have been reported[27] for polynucleotides. Thus, the anionic character and moderately large molecular weight of fraction B could be factors in the gelation of the lysate. When fraction B was treated with periodate and then subjected to the *Limulus* test, the result indicated <4 μg of LPS to be present in 100 mg of the preparation. Mizuno *et al.*[25] reported that the LPS of *Proteus* vulgaris showed only slight activity against solid-type Sarcoma-180 in mice (50 μg/kg gave an inhibition ratio of ~40%). Therefore, it is concluded that the activity of fraction B against Sarcoma-180 is not due to contaminants, but to the alginate-type polysaccharide that is also the active component of fraction A.

In the previous study[2], it was shown that fraction A had no activity against L-1210 leukemia tumour cells *in vitro*, suggesting that its effect *in vivo* may be host-mediated. The possibility that fraction B has similar properties is under investigation.

The M/G ratio (2.78) of fraction B is higher than those (0.3–2.0) of alginates[28,29] from various species, except for that (19) of *Fucus vesicularus*, whereas its molecular weight (33,400) is lower than those reported[21] for alginates. These differences are associated, at least in part, with the antitumour activity of fraction B.

Pyran copolymers (polycarboxylates) show[30] activity against the LSTRA leukemia and Lewis lung carcinoma, and poly(acrylic acid–maleic anhydride), poly(maleic anhydride), and poly(acrylic acid-3,6-endoxo-1,2,3,6-tetrahydrophthalic anhydride) also show[31] activity variously against the Lewis lung carcinoma and Ehrlich ascites-tumour cells. These facts indicate that anionic character is associated with antitumour activity, as could be the case for fraction B.

Moreover, (1→3)-β-D-glucans and β-D-glucans having a preponderance of (1→3) linkages in the main chain show[32] antitumour activity, whereas β-D-glucans containing mainly (1→6) linkages are less active, suggesting that β linkages and unbranched structures may be associated with antitumour activity. Alginates contain[21] β linkages and are unbranched, and these features may be associated with their antitumour activity and with that of fraction B.

Polymannuronate has[33] a twisted conformation and polyguluronate a buckled one; thus, the M/G ratio and, perhaps, the sequence of uronic acids may affect the conformation of alginates and the antitumour activity. Pyran copolymers (polycarboxylates) having molecular weights in the lower part of the range (12,500–52,600) were[34] the more active against the Lewis lung carcinoma, and those in the higher part were the more active against the P815 mastocytoma. Thus, molecular weight can have an important effect on antitumour activity. The relationship between the molecular weight, M/G ratio, and uronic acid sequence and the antitumour activity of alginates is under investigation.

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