SOME STRUCTURAL FEATURES AND VISCOMETRIC PROPERTIES OF THE EXTRACELLULAR POLYSACCHARIDE FROM

Porphyridium cruentum

DARRELL G. MEDCALF, JEFF R. SCOTT, JAMES H. BRANNON, GLENN A. HEMERICK, RONALD L. CUNNINGHAM, JAMES H. CHESSEN, AND JITU SHAH

Department of Chemistry, University of Puget Sound, Tacoma, Washington (U. S. A.)

ABSTRACT

The red alga *Porphyridium cruentum*, when cultured in an artificial sea-water medium, produced a non-dialyzable polymer, which was mainly a sulfated (7.4%) polysaccharide containing glucuronic acid (9%), and xylose and L-galactose (molar ratio 1:0.9). A minor proportion of glucose was found, as well as traces of mannose and rhamnose. On the basis of data from methylation analysis, partial acid hydrolysis, and Smith degradation, the polymer was found to have mainly (1→4)-linkages with branches at position 3 of xylose and positions 2 or 6 of galactose. The glucose and glucuronic acid residues were (1→3)-linked, with some branching at position 6 of glucose. Terminal xylose and galactose were found in equal amounts in the de-sulfated, reduced polysaccharide. 1% Solutions of the polymer were viscous, and showed good pH and temperature stability.

INTRODUCTION

The red alga *Porphyridium cruentum* produces[1] a soluble polysaccharide which contains uronic acid, galactose, xylose, glucose, and sulfate. However, no quantitative work or structural analysis has been reported. A similar polymer is produced[2] by another red alga, *Porphyridium aerugineum*, but no quantitative or structural work has been described.

As part of a research program seeking new uses and sources for marine polymers[3,4], the extracellular polysaccharide from *P. cruentum*, which appeared to have potential for industrial production and utilization, has been studied in some detail.

EXPERIMENTAL

Polymer production and isolation. — The alga, *Porphyridium cruentum* Naeg. (M. B. Allen culture No. R-1.1.1, obtained from M. Doty, Woods Hole, Mass., U. S. A.), was grown in Allen's "SWM" liquid and agar medium, as described by

Hemerick[5]. Algae were grown in 1% CO_2-aerated medium in a 40-litre aquarium lined with polyethylene sheet, and illuminated by fluorescent and incandescent lamps. The temperature was controlled at 20° by cold water circulating in tubing under the polyethylene liner. The culture was stirred by an air immersion pump.

After 14 days, the algal cells were harvested by centrifugation, and the medium was returned to the aquarium and re-inoculated. After a second 14-day growth period, cells were again harvested. The two cell-fractions were combined and freeze-dried. The culture medium was concentrated and dialyzed, and the polymer was isolated by freeze-drying.

General characterization. — Specific optical rotation, sulfate, nitrogen, and uronic acid were determined as previously described[6,7]. Methoxyl values were obtained commercially (Galbraith Laboratories, Knoxville, Tenn., U.S.A.) and the Seliwanoff test was used to detect 3,6-anhydrogalactose[8].

Paper chromatography. — Qualitative and preparative p.c. were performed on Whatman Nos. 1 and 3MM papers, respectively, using *A*, ethyl acetate–pyridine–water (10:4:3); *B*, 1-butanol–acetic acid–water (5:1:2), *C*, 1-butanol–ethanol–water (5:1:4), *D*, ethyl acetate–pyridine–water (8:2:1). Components were detected with either $AgNO_3$–NaOH or *p*-anisidine[9].

Fractionation on DEAE-cellulose[10]. — The polymer (200 mg) was applied to a column containing 20 g of DEAE-cellulose (CO_3^{2-} form), and eluted with 1 litre of H_2O, followed by a gradient elution (0→M) over 1.5 l of $(NH_4)_2CO_3$. The water eluate was collected, and analyzed for carbohydrate by the phenol–sulfuric acid reaction.

Gas chromatography. — G.l.c. was performed on a Pye Series 104 Gas Chromatograph (flame-ionization detector) with nitrogen as the carrier gas. Neutral sugars were analyzed as alditol acetates, using columns (0.3 × 183 cm) of stainless steel packed with 3% of ECNSS-M on Chromosorb W at 190° and 40 ml/min flow-rate, and methylated alditol acetates at 180° and 30 ml/min.

Analysis of neutral sugars. — Neutral sugars were analyzed, after acid hydrolysis with 0.5M H_2SO_4 at 95° for 4–6 h by the alditol acetate procedure[11]. Sugars were identified first by p.c. (solvent *A*). Identification of samples for quantitative analysis by g.l.c. was based on retention times of authentic samples of alditol acetates.

Identification of L-galactose. — A sample (100 mg) of the polymer was hydrolyzed, galactose was isolated by preparative p.c. (solvent *A*; eluted from the paper with H_2O), and the concentration determined by the phenol–sulfuric acid procedure. The specific optical rotation of this solution was then measured, and a sample was also assayed with D-galactose oxidase[12] (Sigma Chemical Company, St. Louis, Mo., U.S.A.),

Desulfation, reduction, and uronic acid identification. — A portion of the polymer was desulfated[13] using 0.3% methanolic HCl. The uronic acid groups then were esterified using diazomethane, and reduced with $NaBH_4$ as described previously[6]. The desulfated and reduced polymer (0.12% of sulfate) was hydrolyzed with acid, and the ratio of neutral sugars was determined by g.l.c.

Partial, acid hydrolysis. — A portion (0.64 g) of polymer was hydrolyzed for 10 h with boiling, saturated, aqueous oxalic acid. After neutralization with barium carbonate, the concentrated solution was placed on a column (19 × 150 mm) of Amberlite IR-45(AcO⁻) resin, and eluted with 2 1 of water followed by a gradient (0 → 2M) of formic acid (2 1); 15-ml fractions were collected. Every tenth fraction was concentrated, and the residue was examined by p.c. (solvent *B*). Fractions 50–90, which contained incompletely resolved acidic fragments, were combined and concentrated, and the residue was fractionated by preparative p.c. (solvent *B*). Five fractions were obtained having R_{GLCA} values of 0.81, 0.88, 0.94, 1.09, and 1.17, respectively. The largest fractions (I and V, 17 mg and 47 mg, respectively) were treated in sequence with 2% methanolic HCl and lithium aluminum hydride[14]. A portion of each product was hydrolyzed, and analyzed for neutral sugars as described above.

Periodate oxidation and Smith degradation. — Periodate oxidation was performed as described previously[6]. Formic acid was determined by titration with 5mM $Ba(OH)_2$. After oxidation was complete, the excess of periodate was reduced with ethylene glycol, the oxidized polymer was reduced with sodium borohydride, and the solution was dialyzed and freeze-dried. A portion of the oxidized polysaccharide was analyzed for neutral sugars after acid hydrolysis. A solution of the remaining material (0.4 g) in 100 ml of 0.13M sulfuric acid was stored at room temperature for 24 h and then poured into 6 vol. of ethanol, and the mixture was kept at 4° overnight. The resulting precipitate was collected by centrifugation, and dissolved in water, and the solution was freeze-dried to give product *P*. The supernatant solution was neutralized with barium carbonate, concentrated, and freeze-dried to give product *S*. *P* and *S* were hydrolyzed, and analyzed for neutral sugars, and *P* was analyzed for uronic acid content and methylated. The methylated sugars were subjected to p.c. and g.l.c., as described below.

Product *S* was examined by p.c. (solvent *A*). Four components were found, and isolated by preparative p.c. (solvent *A*). Components I, II, and IV were hydrolyzed, and analyzed for neutral sugars.

Methylation analysis. — Desulfated, esterified, and reduced polymer (see above) was methylated by the micro sodium hydride–methyl sulfoxide procedure[15]. Complete methylation was indicated by the absence of i.r. absorption for hydroxyl in the methylated product. The methylated polymer was hydrolyzed first with 5 ml of 0.25M H_2SO_4 at 100° for 12 h. After neutralization of the hydrolysate with barium hydroxide and concentration to dryness under reduced pressure, the residue was hydrolyzed with 90% formic acid (10 ml) at 100° for 5 h. The formic acid was removed by evaporation under reduced pressure at 25°, and the resulting syrup was examined by p.c. (solvent *C*). The remaining material was reduced and acetylated[16]. The resulting alditol acetates were separated by g.l.c., and identification was based on the relative retention times given by Björndal *et al.*[17], using the alditol acetates of 3-*O*-methyl-D-xylose and 2,3,4,6-tetra-*O*-methyl-D-glucose as standards.

Site of ester sulfate. — When a portion of the polymer was treated with

alkali[18], 3,6-anhydrogalactose moieties were not formed. In order to determine whether xylose residues contained ester sulfate, the polymer (20–40 mg) was treated in sequence with sodium borohydride and sodium hydroxide[19]. The resulting product was isolated by freeze-drying after dialysis, hydrolyzed, and then examined by p.c. (solvents *A* and *D*). A second portion of *P. cruentum* polymer was treated with base and isolated as described above. After thorough drying over P_2O_5 *in vacuo*, the polymer was treated with sodium methoxide in methanol[19] and then hydrolyzed with 0.5M sulfuric acid, and examined by p.c. (solvent *C*).

Viscosity measurements. — Viscosity studies were done with a Brookfield Viscometer, using a No. 3 spindle at 20 r.p.m. For temperature, pH, and salt-effect studies, 2 g of polymer were added to 198 ml of water, and the dispersion was stored at room temperature for 15 h prior to viscosity measurements. The same hydration period was also used for the samples at other concentrations.

RESULTS AND DISCUSSION

The yield of extracellular polymer was 0.47 g/l of culture medium. No attempt was made to optimize the growth conditions for maximal yield of polysaccharide, which was isolated as an easily hydrated, slightly tan material. Complete dissolution or dispersion in water took several hours. In addition to the extracellular polymer, 1.7 g/l of freeze-dried cells were isolated. A similar water-soluble polymer was extracted from these cells in good yield[3].

Nitrogen analysis indicated the presence of 5.6% of protein (N × 6.25), so that the polymer isolated was mainly polysaccharide; other analytical data are shown in Table I.

TABLE I

GENERAL CHARACTERIZATION OF *P. cruentum* POLYSACCHARIDE

			Molar ratio
$[\alpha]_D^{25}$ (degrees)	+56	Xylose	1.0
Sulfate (%)	7.2	Galactose	0.9
Uronic acid (%)	9	Glucose	0.4
OMe (%)	0.6	Rhamnose	trace
3,6-Anhydrogalactose	negative	Mannose	trace

The uronic acid was shown to be glucuronic acid by comparison of the neutral sugar ratio before and after methyl esterification and reduction with lithium aluminum hydride. The amount of glucose increased significantly in relation to the other neutral sugars. By comparison with previous work[20], it is probable that the xylose, glucose, and glucuronic acid were all D isomers. However, both D- and L-galactose are common in red algae. A sample of galactose (15 mg), isolated by preparative p.c. from the polymer after acid hydrolysis, had $[\alpha]_D$ −63°, indicative of the preponderance of the

L isomer ($[\alpha]_D$ −80°). Assay of the galactose with D-galactose oxidase indicated the presence of 90% of L- and 10% of D-galactose. This is an unusual observation, as D-galactose generally preponderates when D and L forms are found in a polymer[20].

The heterogeneity of the polymer was investigated by fractionation on DEAE-Cellulose (CO_3^{2-} form). Elution with water and then with ammonium carbonate gave several broad peaks, with the largest fractions eluted from the column with H_2O and M $(NH_4)_2CO_3$. These results are sumarized in Table II. Whereas some separation into fractions containing various amounts of uronic acid occurred, the neutral sugar composition changed very little. The data suggested that there was some heterogeneity in the sample, but that the material was a single general type, namely, a sulfated glucuronoglucogalactoxylan. The structural characterization was continued on the unfractionated material.

.TABLE II

FRACTIONATION OF POLYMER ON DEAE-CELLULOSE (CO_3^{2-} FORM)

Fraction	Yield[a] (%)	Uronic acid (%)	Ratios Xyl:Gal:Glc
Original	—	9	1.0:0.9:0.4
H_2O	23	6	1.0:0.7:0.4
0.5M $(NH_4)_2CO_3$	15	[b]	1.0:0.9:0.7
M $(NH_4)_2CO_3$	25	22	1.0:0.7:0.4

[a]Total recovery of material was 97%. [b]Not determined.

The primary structural features of the polymer were determined by methylation analysis of the desulfated, esterified, and reduced polymer. These results are shown in Table III. Incomplete resolution of some of the peaks for methylated sugars in g.l.c. made quantitative determination of molar ratios difficult. The values shown in Table III are therefore approximate, but give a good estimate of the composition. Xylose and galactose, but not glucose, were found as non-reducing terminal residues. The remaining xylose residues were (1→4)-linked, with a small amount of C-3 branches as suggested by the formation of 2-O-methylxylose. However, this result could also be due to traces of xylose 3-sulfate in the desulfated polymer. Galactose was also (1→4)-linked, with branches at both C-3 and C-6 (indicated by the formation of 2,6-di-O-methyl- and 2,3-di-O-methylgalactose, respectively). With the procedure used in this work, glucose and glucuronic acid cannot be distinguished. However, both must be (1→3)-linked. The 2,4-di-O-methylglucose must come from glucose in the original polymer, which is an indication of branches at position 6 of glucose. The glucuronic acid residues appeared to be unbranched.

The data for periodate oxidation and Smith degradation are shown in Table IV. There was one end-group for every five sugar units, as indicated by the release of formic acid. The total uptake of periodate suggested that an additional 20% of the sugar units were also periodate-labile. The xylose residues were most susceptible, and

TABLE III

METHYLATION ANALYSIS OF THE POLYSACCHARIDE

Methylated sugars	Approximate molar ratio
Xylose	
2,3,4-tri-*O*-methyl	1
2,3-di-*O*-methyl	0.5
2-*O*-methyl	trace
Galactose	
2,3,4,6-tetra-*O*-methyl	1
2,3,6-tri-*O*-methyl	0.4
2,6-di-*O*-methyl	0.2
2,3-di-*O*-methyl	0.2
Glucose	
2,4,6-tri-*O*-methyl	0.2
2,4-di-*O*-methyl	0.2

the glucose residues were almost all stable. This was indicated by the methylation analysis and confirmed by the neutral sugar data on the periodate-oxidized polymer. The relative amount of glucose increased in relation to galactose and xylose, and the amount of galactose also increased in relation to xylose. The periodate uptake in the desulfated polymer was 0.61 mol/mol of hexose, suggesting that most, but not all, of the sulfate ester was attached to primary hydroxyl groups.

TABLE IV

PERIODATE OXIDATION OF THE POLYSACCHARIDE

	Original polymer	Periodate-oxidized polymer	S	P
Periodate uptake (mol/mol of hexose)	0.56	—	—	—
Formic acid (mol/mol of hexose)	0.2	—	—	—
Yield (%)	—	95[a]	38[b]	62[b]
Neutral sugars				
Xylose	1.0	1.0	1.0	1.0
Galactose	0.9	2.3	0.3	4.5
Glucose	0.4	1.4	0.5	0.7

[a]Based on amount of polymer treated with periodate. [b]Based on the amount of periodate-oxidized polymer hydrolyzed.

Mild, acid hydrolysis of the periodate-oxidized polysaccharide, followed by alcohol precipitation, yielded two fractions *P* and *S* of relatively low molecular weight. This feature was indicated for *P* by a large increase in end-groups on methylation analysis. These data suggest that there were no large periodate-resistant sections in the polysaccharide. However, the preponderance of galactose in *P* compared to *S* indicated that some portions of the molecule contained significant periodate-resistant

segments of C-3 branched galactose residues, along with (1→3)-linked glucose and glucuronic acid residues.

Fraction S contained 4 components separable by p.c., but no product appeared to preponderate and all were isolated in very low yield. The R_{XYL} (solvent A) values were 0.20, 0.43, 0.63, and 0.78, respectively. After isolation by p.c., each compound was hydrolyzed with acid and the products were identified by p.c. and g.l.c. The fraction with R_{XYL} 0.20 was an oligosaccharide containing threitol, xylose, galactose, and glucose. The component with R_{XYL} 0.43 contained threitol, xylose, and galactose, whereas that with R_{XYL} 0.78 contained glycerol and xylose. The fourth component was lost. The quantitative g.l.c. data were inconclusive because of the very small quantities available. These data confirmed that the polymer was broken into small fragments by the Smith degradation, and suggested a relatively random distribution of periodate-labile and -resistant units. The presence of glucose, galactose, and xylose in the polymer was confirmed by these results.

The result of partial hydrolysis with acid also indicated that xylose, galactose, and glucuronic acid were part of the same molecule. Two of the five components were analyzed for neutral sugars after conversion of the glucuronic acid into glucose. Component I (R_{GLCA} 0.81) contained xylose (1.0), galactose (1.0), and glucose (1.6). The data suggest a tetrasaccharide containing xylose, galactose, and glucuronic acid. Component V (R_{GLCA} 1.17) contained xylose (1.0), galactose (0.8), and glucose (0.7). This suggests a trisaccharide containing xylose, galactose, and glucuronic acid. In each of these products, the glucose may have come from glucose or glucuronic acid. However, the much larger content of glucuronic acid in the polymer plus the method of isolation suggest the acid as the most likely source.

The i.r. spectrum (KBr) of the polymer contained a broad band at 1240 cm^{-1} indicative of sulfate esters[21], and a relatively weak absorption at 820 cm^{-1}. There was no absorption at 850 cm^{-1}, indicating that little sulfate was present on positions 2 or 3 of galactose or xylose[22]. The band at 820 cm^{-1} indicated primary sulfate ester, probably involving galactose (glucose is also a possibility)[23]. Chemical analysis using the procedures of Rees[18] and Percival and Wold[19] were inconclusive. Treatment of the polymer with base gave no 3,6-anhydrogalactose, suggesting that the 6-sulfated galactose residues were also substituted at position 3. The base-treated polymer gave some arabinose when hydrolyzed, indicating the presence of xylose 2- or 3-sulfate[19]. However, the position of the sulfate group could not be confirmed by treating the epoxide polymer with methoxide. This problem has been encountered before when small amounts of polymer were used[24].

There are similarities and differences when the structure of the *P. cruentum* polymer is compared to that of the extracellular polymer from the unicellular, red alga *Rhodella maculata*[25].

The *P. cruentum* polymer formed very viscous solutions at the 1% level. Since the polymer was easily isolated in reasonable quantity, potential for use as an industrial gum was suggested. Therefore, the viscometric properties were investigated. These results are summarized in Figs. 1–4. The viscosity of a 1% dispersion of the
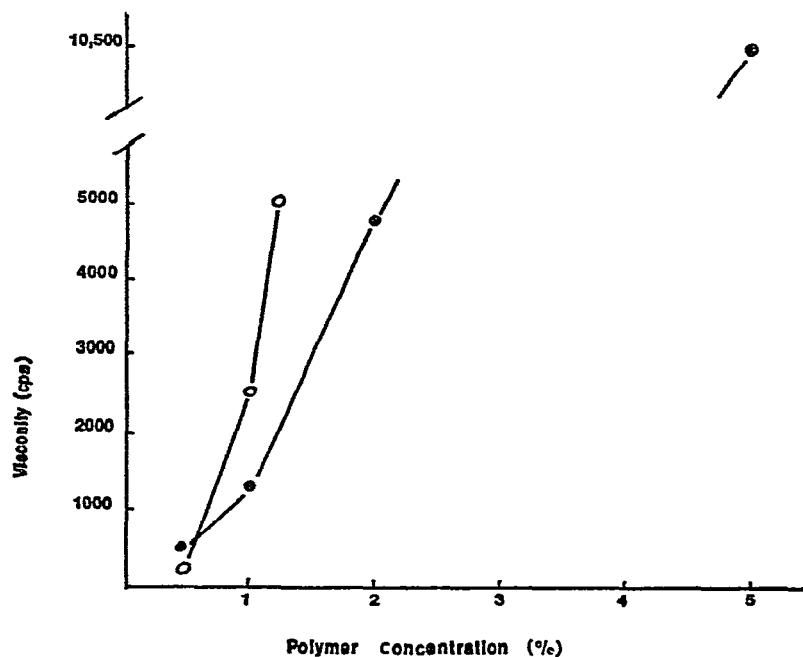
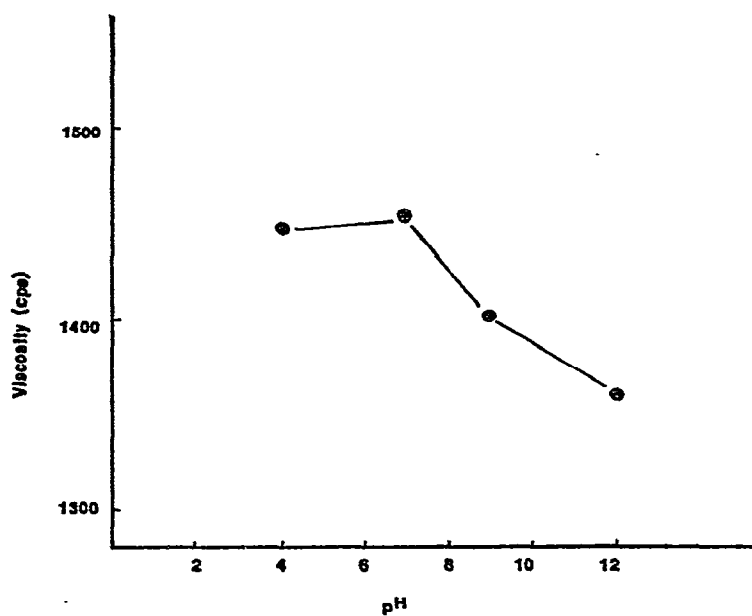Fig. 1. Brookfield viscosity of *P. cruentum* polymer: O, guar gum; ⊝, *P. cruentum.*

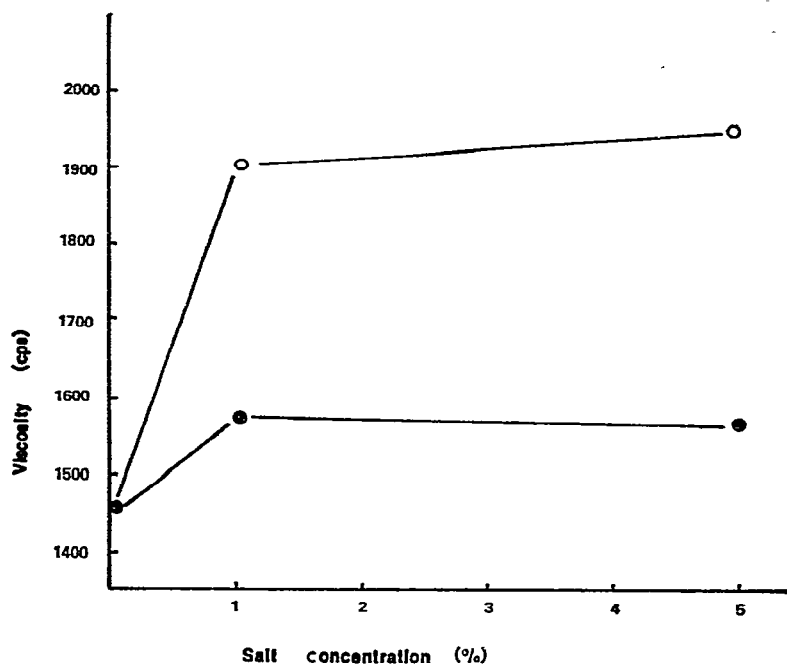Fig. 2. Effect of pH on polymer viscosity (1% dispersion).

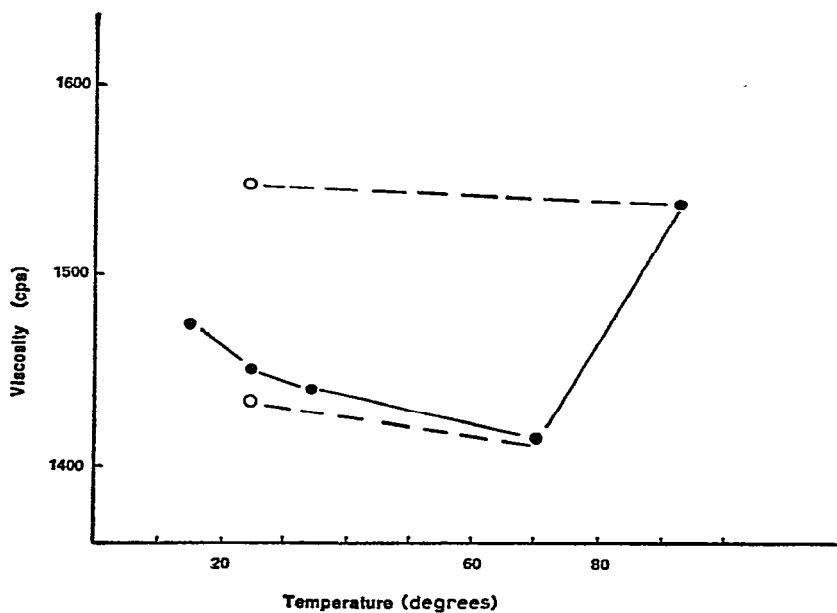Fig. 3. Effect of salt on polymer viscosity (1% dispersion): ●, CaCl₂ ; ○, NaCl.

Fig. 4. Effect of temperature on polymer viscosity (1% dispersion). Dotted lines indicate the viscosity of the solution when cooled back to 25°.

polymer showed very good pH-stability and compatibility with mono and divalent salts (Figs. 2 and 3). Fig. 4 indicates the temperature stability of the polymer dispersion. Little change was evident until the polymer was heated at 90°, where the viscosity increased and was retained when the dispersion was cooled to 25°. This result indicated that more-complete hydration of the polymer had occurred at 90°. No heat degradation was detected.

These data suggest that the *P. cruentum* polymer may have properties suitable for industrial use as a thickening agent.

REFERENCES

1 R. F. JONES, *J. Cellular Comp. Physiol.*, 60 (1962) 61–64.
2 J. RAMUS, *J. Phycol.*, 8 (1972) 97–111.
3 D. G. MEDCALF, J. H. BRANNON, J. R. SCOTT, J. LEWIN, G. G. ALLAN, AND R. E. NORRIS, *Proc. Int. Seaweed Symp. 8th*, (1974) in press.
4 G. G. ALLAN, J. LEWIN, AND P. G. JOHNSON, *Botan. Marina*, 15 (1972) 102–108.
5 G. HEMERICK, in JANET R. STEIN (Ed.), *Handbook of Phycological Methods–Culture Methods and Growth Measurements*, Cambridge University Press, 1973, p. 255–266.
6 D. G. MEDCALF, C. F. ROOT, C. L. CRANEY, D. MUKHOPADHYAY, C. K. MILLER, AND W. D. HOPEWELL, *Proc. Int. Seaweed Symp., 7th, University of Tokyo*, 1972, p. 541–547.
7 D. G. MEDCALF, T. LIONEL, J. H. BRANNON, AND J. R. SCOTT, *Botan. Marina*, (1975) in press.
8 D. M. BOWKER AND J. R. TURVEY, *J. Chem. Soc., C*, (1968) 983–988.
9 L. HOUGH AND J. K. N. JONES, *Methods Carbohyd. Chem.*, 1 (1962) 21–31.
10 I. R. SIDDIQUI AND P. J. WOOD, *Carbohyd. Res.*, 16 (1971) 452–454.
11 L. G. BORCHARDT AND C. V. PIPER, *Tappi*, 53 (1970) 257–260.
12 H. ROTH, S. SEGAL, AND D. BERTOLI, *Anal. Biochem.*, 10 (1965) 32–52.
13 Q. N. HAQ AND ELIZABETH PERCIVAL, in E. G. YOUNG AND J. L. McLACHLAN (Eds.), *Proc. Int. Seaweed Symp., 5th*, Pergamon Press, London, 1966, p. 261–269.
14 J. J. O'DONNELL AND E. PERCIVAL, *J. Chem. Soc.*, (1959) 2168–2178.
15 H. BJÖRNDAL AND B. LINDBERG, *Carbohyd. Res.*, 10 (1969) 79–85.
16 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801–1804.
17 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
18 D. A. REES, *J. Chem. Soc.*, (1961) 5168–5171.
19 E. PERCIVAL AND J. K. WOLD, *J. Chem. Soc.*, (1963) 5459–5468.
20 E. PERCIVAL AND R. H. McDOWELL, *Chemistry and Enzymology of Marine Algal Polysaccharides*, Academic Press, New York, 1967, p. 191.
21 J. R. TURVEY, *Advan. Carbohyd., Chem.*, 20 (1965) 183–212.
22 A. G. LLOYD AND K. S. DODGSON, *Biochim. Biophys. Acta*, 46 (1961) 108–115.
23 A. G. LLOYD AND K. S. DODGSON, *Biochim. Biophys. Acta*, 46 (1961) 116–120.
24 J. LOVE AND E. PERCIVAL, *J. Chem. Soc.*, (1964) 3338–3350.
25 L. V. EVANS, M. E. CALLOW, E. PERCIVAL, AND V. FAREED, *J. Cell Sci.*, 16 (1974) 1–21.