ELSEVIER

Carbohydrate Polymers 50 (2002) 183–189

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Characterization of the extracellular polysaccharide of *Porphyridium* sp.: molecular weight determination and rheological properties

S. Geresh*, I. Adin, E. Yarmolinsky, M. Karpasas

The Institute for Applied Biosciences, Ben-Gurion University of the Negev, P.O. Box 653, 84105 Beer-Sheva, Israel

Received 8 October 2001; revised 20 December 2001; accepted 10 January 2002

Abstract

The molar mass of the extracellular polysaccharide produced by the red microalga *Porphyridium* sp. was determined by gel permeation chromatography (GPC) followed by multiangle laser light scattering (MALLS). Two different disaggregation treatments were used prior to the measurement: (1) sonication and (2) incubation in an aqueous solution of tris(ethylenediamine)cadmium dihydroxide (cadoxen) followed by GPC analysis using a 50% cadoxen solution in the chromatographic mobile phase. The average molar mass was found to be 2.3×10^6 g/mol with the size distribution lying between 2×10^5 and 4×10^6 g/mol. The viscoelastic properties of aqueous solutions of the polysaccharide before and after sonication were evaluated by rheological methods. It was found that the viscosity of the solutions decreased dramatically with increasing sonication time. Changes in the mechanical spectra of aqueous polysaccharide solutions as a result of sonication over a range of frequencies were reflected in the inversion of the ratio of the storage modulus G' to the loss modulus G'', which indicated a shift from a 'weak gel' to a liquid-like state. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Gel permeation chromatography/multiangle laser light scattering; Polysaccharide; Cadoxen; Sonication; *Porphyridium* sp; Rheology

1. Introduction

The red microalga *Porphyridium* sp. is encapsulated within a sulfated polysaccharide, which has a wide range of promising industrial and medicinal applications (Geresh & Arad (Malis), 1991; Geresh, Mamontov, & Weinshtein, 2002). Structural analysis of this extracellular polysaccharide—as one of the steps towards introducing it into various uses—must include physicochemical characterization. In this context, the importance of determining the rheological properties and molecular weight of the biopolymer lies in the influence of these parameters on its function and behavior.

Characterization of the polysaccharide of *Porphyridium* sp. regarding molecular weight determination has been a particularly challenging task because of a number of factors: the relative multitude of monosugars (Geresh, Lupescu, & Arad (Malis), 1992; Heaney-Kieras & Chapman, 1976; Medcalf, Scott, Brannon, Hemerick, Cunningham, Chessen, & Shah, 1975; Percival & Foyle, 1979; Ramus, 1973); the resistance of the biopolymer to enzymatic digestion (Arad (Malis), Keristovsky, Simon, Barak, & Geresh, 1993a; Simon, Geresh, & Arad (Malis), 1993); the high molecular weight and high apparent viscosity of the polymer (Eteshola, Gottlieb, & Arad (Malis), 1996; Geresh & Arad (Malis), 1991) and the tendency of the molecules to aggregate in solution, as observed during solubilization in water and viscosity measurements. The apparent molecular mass of the polysaccharide has previously been estimated to be 5–7×10^6 Da by size-exclusion chromatography (Arad (Malis) et al., 1993a; Arad (Malis), Dubinsky, & Simon, 1993b; Simon et al., 1993). However, this technique tends to neglect some of the properties of this polysaccharide, such as aggregation and polyelectrolyte nature, which might influence the results. All our attempts to apply chromatographic techniques to the analysis of untreated polysaccharide solutions did not yield satisfactory results, probably as a result of inter-chain interactions in solution, which also influenced the rheological characteristics. It has previously been suggested that the analysis of such polymer complexes could be facilitated by disaggregation in solvents such as DMSO (Bello-Perez, Roger, Baud, & Colonna, 1998), urea (Fang, Qinag, Hu, Wang, & Cui, 2001; Maeda, Watanabe, Chihara, & Rokutanda, 1988; Narui, Takahashi, Kobayashi, & Shibata, 1980) or tris(ethylenediaminecadmium dihydroxide) (cadoxen) (Sato, Norisuye, & Fujita, 1985; Schwald & Bobleter, 1988; Yevlampieva, Pavlov, & Rjumtsev, 1999; Zhang, Ding, Zhang, Zhu, & Zhou, 1997). Another powerful tool that has been proposed

---

* Corresponding author. Tel.: +972-8646-1903; fax: +972-8647-7675.
E-mail address: geresh@bgumail.bgu.ac.il (S. Geresh).

PII: S0144-8617(02)00019-X

Table 1
Characteristics of polysaccharides

Batch No.	Carbohydrate content (%)[a]	Sugar composition (%)[b]			Ash (wt%)
		Xylose	Galactose	Glucose	
178	55	44.5	29.1	26.4	8.6
168	56	44.7	30.2	25.1	10.5
158	57	46.2	29.5	24.3	9.2

[a] Determined as described by Dubois et al.

[b] Determined by GC.

for the disaggregation of macromolecules, especially synthetic polymers, is exposure to ultrasound radiation (Basedow & Ebert, 1977).

In this paper, we present the results of a rheological study of the polysaccharide of *Porphyridium* sp. exposed to ultrasound radiation for various times (in aqueous buffer) and molecular weight determination after exposure of the polysaccharide to ultrasound radiation or cadoxen solution.

2. Materials and methods

2.1. Polysaccharide production

The extracellular polysaccharide of *Porphyridium* sp. (UTEX 6371) was obtained as previously described (Cohen & Arad (Malis), 1989; Jones, 1962). The culture medium was centrifuged (Sorvall 4C-5C, 20–30 min at 4–8 °C) and the supernatant containing the polysaccharide was concentrated using an ultrafiltration system (MaxCell® cartridges produced by A/G Technology Corp., 0.45 μm, 2.5 m^2). Two batches of polysaccharide, designated 158 and 168, were used for the ultrasonication experiments and a third batch, designated 178 was used for the analysis in cadoxen solution. The three batches were similar in carbohydrate content and sugar composition, as shown in Table 1.

Carbohydrates were determined colometrically with a Jasco UV/VIS spectrophotometer by means of the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sugar composition was determined by gas chromatography with a Hewlet Packard 5890 GC equipped with an FID detector on a DB-1 capillary column (30 m, 0.25 mm id) from J & W Scientific, with helium as the carrier gas. Samples of polysaccharide for GC analysis were prepared as follows. A 10 mg sample of dry polysaccharide was hydrolyzed with trifluoroacetic acid (2 M, 2.5 ml) at 100 °C. After cooling, toluene (1 ml) was added, and the solvent and excess trifluoroacetic acid were removed by evaporation, followed by flushing with nitrogen. Then, a solution of sodium borohydride (27.5 mg) and myoinositol (4 mg) in ammonium hydroxide (1 M, 0.25 ml) was added and the reaction mixture was stirred for 1 h at room temperature. Thereafter, 1 ml of 10% glacial acetic acid in methanol was added. The solvent was removed by flushing with nitrogen. Pyridine (0.1 ml) and acetic anhydide (0.1 ml) were added to the remaining mixture, which was then heated at 120 °C for 20 min. The acetylated sample was extracted with CH_2Cl_2 evaporated to dryness and redissolved in dry acetone.

2.2. Ultrasonication

Polysaccharide solutions (1% w/v) were sonicated in a Sonics & Materials microprocessor-controlled high-intensity 600 W Ultrasonic processor, equipped with a 1/8″ tapered microtip. Each of seven samples taken from batch 158 was processed separately at pH 5.5 for 10, 30 s, 1, 2, 8, 20 or 60 min. An additional four samples from the same batch were processed in the same way (10 s, 2, 20, 60 min) at pH 3 and three samples (10 s, 2, 20 min) were sonicated at pH 9. Samples taken from batch 168 were then processed in the same way.

2.3. Preparation of cadoxen

A fresh cadoxen solution was prepared each day as follows. An aqueous solution of ethylenediamine (33% v/v) was saturated with cadmium oxide (CdO, FLUKA) at 0 °C under vigorous stirring and then stored at 4 °C for 16 h. Thereafter, the solution was centrifuged at 10,000 rpm for 20 min and the supernatant was filtered through a 0.2 μm filter.

2.4. Rheological measurements

Rheological measurements were performed on a Carri-Med CSL-50 controlled-stress rheometer (Carri-Med Instruments Ltd, Dorking, UK), operated in cone-plate mode (cone angles 1 and 4° with diameters of 60 and 40 mm, respectively). Small-amplitude oscillatory shear experiments (0.1–10 Hz) were performed within the linear viscoelastic limit. Frequency scans were performed at the lowest stress possible to prevent damage to the sample. The linearity of response was monitored continuously to ascertain linear viscoelasticity.

2.5. GPC–MALLS system

Samples were separated on a chromatographic system comprising a Waters 606 pump followed by two PSS

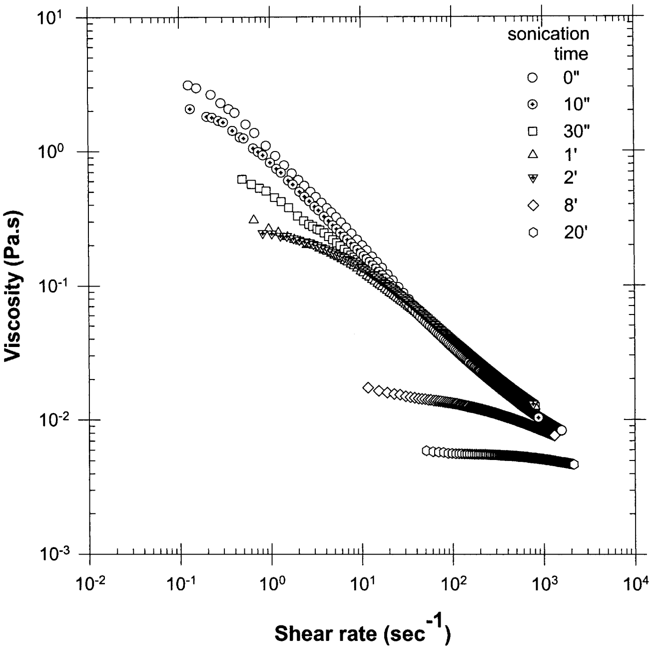

Fig. 1. Effect of sonication time on steady shear viscosity of a 1% (w/v) aqueous *Porphyridium* sp. polysaccharide solution.

Suprema gel permeation columns connected in series. Column description: dimensions 300 × 8 mm^2, particle size 10 μm, porosity 3000 and 10,000 Å. Flow rate was 0.5 ml/min. The columns were kept at a constant temperature of 25 °C inside a Techlab K-4 controlled oven. The chromatographic system was attached to a Dawn DSP (Wyatt Technology Corporation) multiangle laser light scattering (MALLS) photometer equipped with a He/Ne laser working at 632.8 nm, a K5 refraction cell and 18 detectors at angles 14–163°. Concentration was monitored by a calibrated interferometric refractometer Optilab DSP (Wyatt Technology Corporation). Data processing and molar mass calculation were performed with Wyatt ASTRA software version 4.7. Each sample was injected three times to ensure reproducibility. The d*n*/d*c* of the polysaccharide, measured with the Optilab DSP, controlled by Wyatt d*n*/d*c* software, was found to be 0.14 ml/g (aqueous buffer). Aqueous buffer solutions were prepared from ultra pure water (0.055 μs/cm, USF SERAL Purelab RO75 followed by USF SERAL Purelab UV) to which was added 0.1 M $NaNO_3$, 0.02% (w/v) NaN_3 and 10 mM imidazole. The buffer was titrated with HNO_3 (70 and 7% v/v) to pH 7 and filtered through a 0.1 μm filter (Gelman Sciences VacuCap 60).

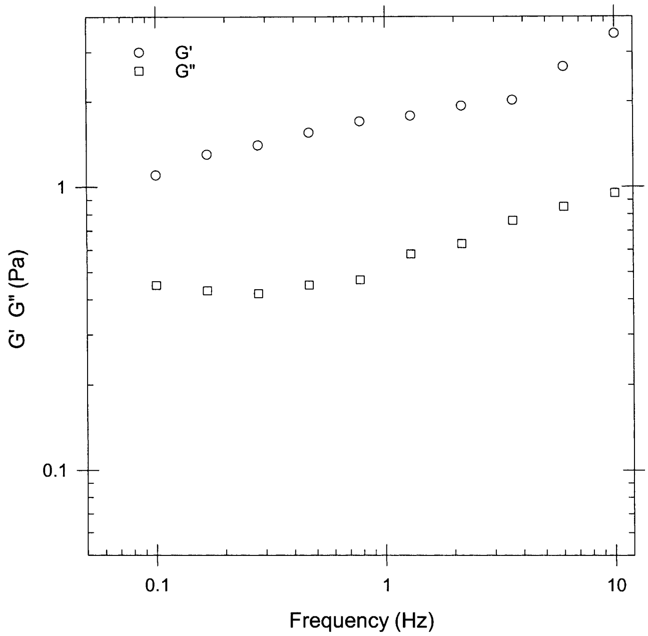

Fig. 2. Mechanical spectra of 1% (w/v) aqueous solution of *Porphyridium* sp. polysaccharide before sonication.

For the experiments in cadoxen solution, the polysaccharide was allowed to stand overnight in a 50% w/v solution of cadoxen before the analysis. Cadoxen solution (50% w/v) was also used as the chromatographic mobile phase. A mobile phase refractive index of $n_0 = 1.3587$ was used for light scattering measurements based on literature values (Sato et al., 1985; Schwald & Bobleter, 1988; Yevlampieva et al., 1999; Zhang et al., 1997). The d*n*/d*c* of the polysaccharide solution in 50% cadoxen was set to 0.1525 on the basis of our measurements and on literature values (Schwald & Bobleter, 1988; Yevlampieva et al., 1999).

3. Results and discussion

3.1. Steady shear viscosity

The large deformation steady shear results are presented as plots of viscosity (η) vs. shear rate (γ). For sonication

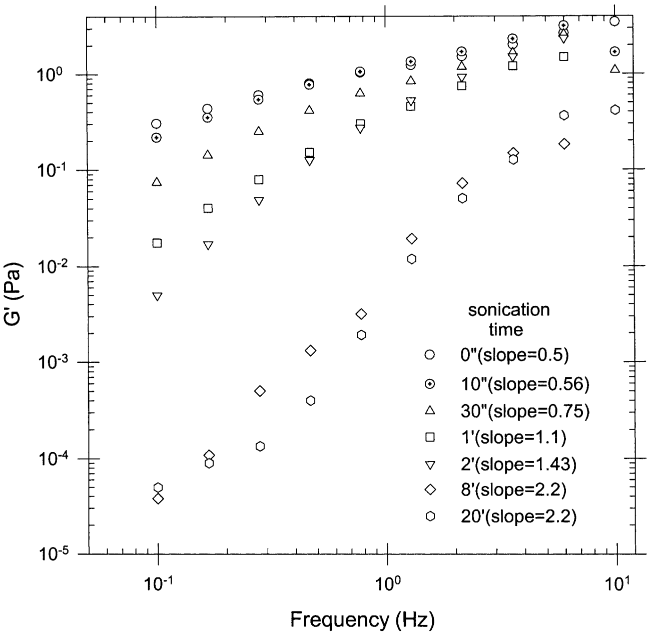

Fig. 3. Elastic response (storage modulus, G') for 1% (w/v) aqueous solution of *Porphyridium* sp. polysaccharide as a function of sonication time.

times in the range 0–20 min, the flow curves of a 1% (w/v) aqueous *Porphyridium* sp. polysaccharide solution were strongly dependent on the sonication time (Fig. 1). All samples showed marked shear-thinning behavior, i.e. decreasing viscosity with increasing shear rate under steady shear conditions. The degree of shear-thinning decreased as the sonication time increased. The measured viscosity decreased rapidly as a function of sonication time.

A power-law relationship ($\eta \cong \gamma^{-0.9}$) was observed for a plot of log η vs. log γ for the untreated 1% aqueous polysaccharide solution and for a solution that had been exposed to 10 s of sonication. This is a typical power-law relationship of a structured material. Typical random coil polysaccharide solutions and non-structured systems usually show exponent values of -0.74 or less for this power-law (Lapasin & Pricl, 1995). Such values were observed for samples that had been sonicated for longer than 20 s. These results are compatible with the observed decrease in molar mass as a function of sonication time, as discussed later. The effect of the pH (pH 3, 5.5 and 9) of polysaccharide

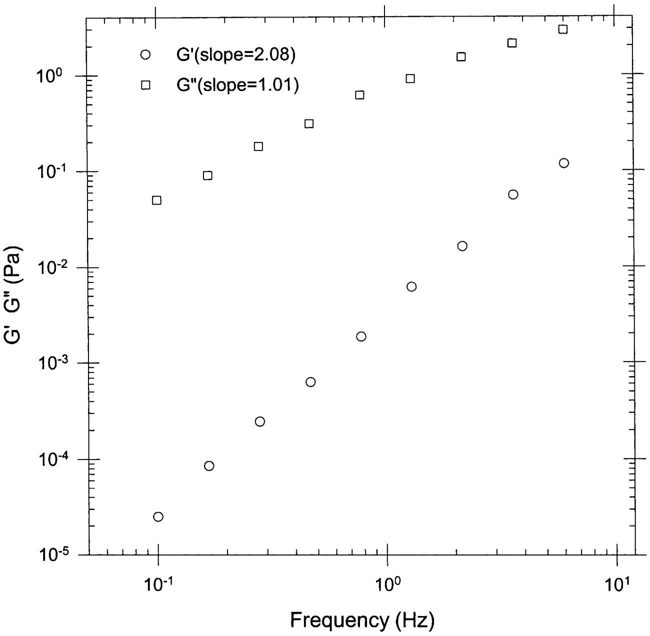

Fig. 4. Mechanical spectra of 1% (w/v) aqueous solution of *Porphyridium* sp. polysaccharide after 60 min of sonication.

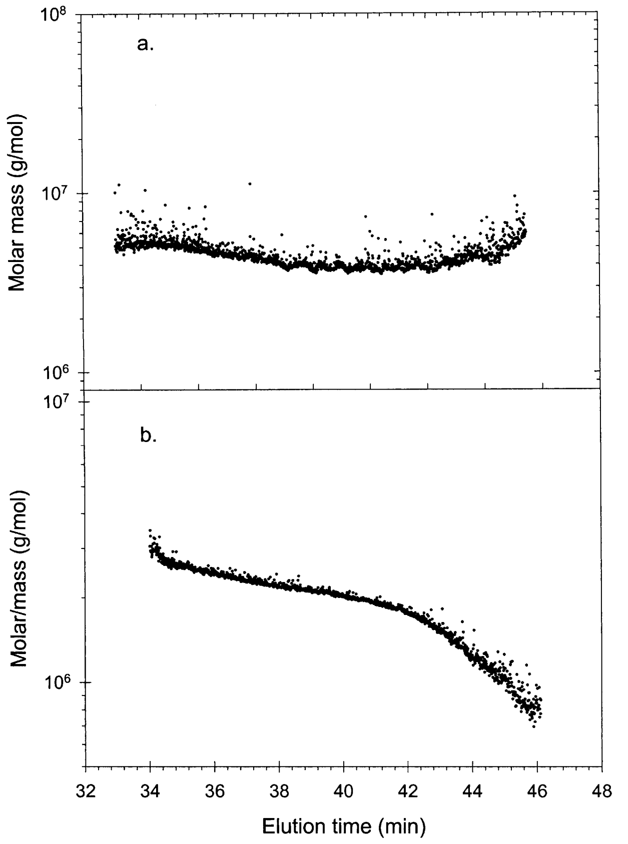

Fig. 5. Molar mass as a function of elution time for: (a) native *Porphyridium* sp. polysaccharide solution; (b) after 5 s of sonication.

solutions during sonication was also tested. Changes in the pH values did not affect the viscosity, as shown by steady shear viscosity measurements.

3.2. Small deformation oscillatory measurements

Small deformation oscillatory measurements are presented in terms of the storage modulus G' (elastic response) and the loss modulus G'' (viscous response) as functions of the angular frequency, G' being used as the primary indicator of a gel-like (structured) system. Fig. 2 shows the frequency dependence of the rheological response on a 1% (w/v) aqueous *Porphyridium* sp. polysaccharide solution before sonication. For the native polysaccharide solution, the value of G' exceeded that of G'' with both moduli showing only a slight dependence on frequency. This behavior is typical of a structured system. As sonication time increased, G' fell dramatically over the frequency range tested (Fig. 3). After 60 min of sonication, the mechanical spectrum of *Porphyridium* sp. polysaccharide resembled that of a polymer solution with G'' higher than G' (Fig. 4). The slopes of log G' and log G'' vs. logω were calculated and found to be 2.08 and 1.01, respectively. These values point as expected to a solution of non-overlapping disordered coils (Lapasin & Pricl, 1995).

3.3. Molecular weight measurements

In our determinations of molecular weight with the GPC–MALLS system, the degree of size separation obtained in each experiment was verified by means of the molar mass vs. elution time diagram, produced by the ASTRA software (Wyatt Technology Co., USA). Usually, size-exclusion chromatography results in the higher molecular weight fragments coming out before the smaller ones, which produces a clear negative slope for the earlier-mentioned diagram. Attempts to determine the molecular weight of the native polysaccharide, without prior disaggregation treatment, were only partially successful, probably because aggregation of the polysaccharide molecules in solution prevented size separation. A very poor slope was obtained for untreated polysaccharide, as can be seen in Fig. 5a.

Attempts to use a disaggregation medium such as DMSO or urea solution, as previously reported (Bello-Perez et al., 1998; Fang et al., 2001), were unsuccessful. Ultrasound radiation, however, proved to be a successful means of breaking up the macromolecules in solution (Basedow & Ebert, 1977). It is possible that the first short sonication

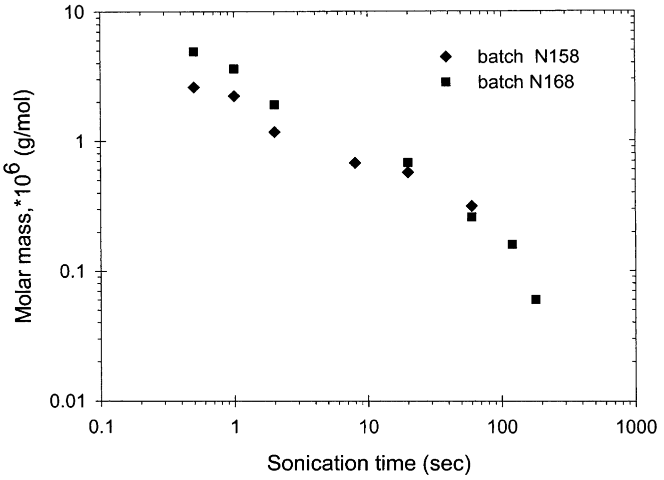

Fig. 6. Effect of sonication time on the average molar mass of *Porphyridium* sp. polysaccharide solution.

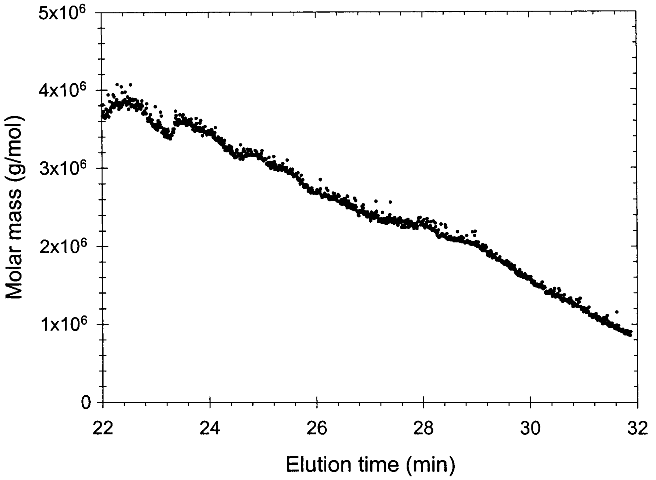

Fig. 7. Molar mass as a function of elution time for *Porphyridium* sp. polysaccharide in cadoxen solution (50% v/v).

periods (5–30 s) used by us may have caused minor chain shortening, but they were instrumental in affording the first reliable measurement of the molecular weight of the polysaccharide.

Chromatographic separation improved after a minimum of 5 s of sonication (Fig. 5b), but longer sonication times caused degradation, as was manifested by a decrease in molecular weight (Fig. 6). Also, the pH of the polysaccharide solution had no effect on the degradation process of the biopolymer during sonication. For all samples for which chromatographic separation was reasonable, results for the second and third injections were reproducible within 4%—and often within less than 2%. A direct correlation was observed between the time of ultrasound processing and the degree of reinjection reproducibility.

The lowest chain size obtainable by ultrasound radiation is reflected in the limiting value of the degradation process M_{lim} (Price, West, & Smith, 1994). After long sonication time, we found M_{lim} to be about 60,000 Da for the *Porphyridium* sp. polysaccharide. The results obtained from gel permeation chromatography (GPC) analysis of sonicated aqueous solutions of the polysaccharide of *Porphyridium* sp. thus provide additional proof for the initial state of aggregation of this biopolymer in solution.

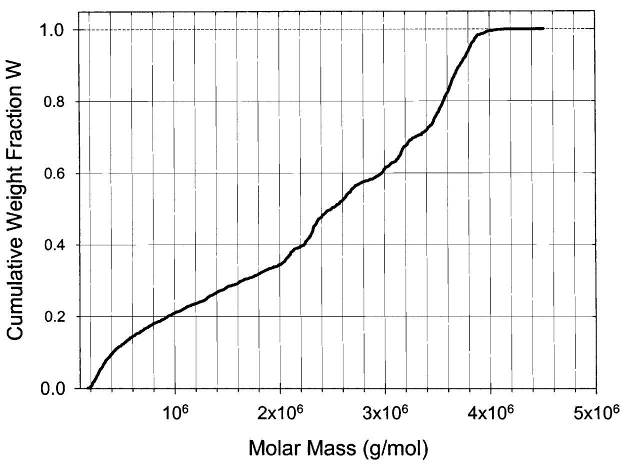

Fig. 8. Cumulative molar mass distribution of *Porphyridium* sp. polysaccharide in cadoxen solution (50% v/v).

3.4. Measurements in cadoxen

The other disaggregation treatment that was applied comprised exposure to cadoxen medium prior to and during GPC–MALLS analysis. Chromatographic measurements in cadoxen have previously been used (Sato et al., 1985; Yevlampieva et al., 1999; Zhang et al., 1997) to aid in the analysis of complex polysaccharide solutions that exhibit severe aggregation. In our experiments, we used gradually increasing concentrations of cadoxen in the polysaccharide solution. Only when the final concentration of cadoxen reached 50% (v/v) was a good separation plot obtained (Fig. 7). The good size separation is evident from the slope of the plot of molar mass vs. elution time, which was not obtained in the analysis of the native material. Good reproducibility was observed for the calculated molar mass of successive injections (<4%). The size distribution obtained for the molar mass lay between 2×10^5 and 4×10^6 g/mol (Fig. 8). For approximately 50 wt% of the sample, the molar mass exceeded 2.5×10^6 g/mol and for approximately 25 wt% of the sample, it fell below 1×10^6 g/mol. The average calculated molar mass was 2.3×10^6 g/mol. The observed distribution of the radii of gyration—indicative of the size of the macromolecule—fell between 75 and 350 nm, and the average radius was 180 nm.

Acknowledgements

The authors wish to thank Prof. Shoshana (Malis) Arad for supplying the *Porphyridium* sp. polysaccharide for this work and Ms Dorit Van-Moppes and Ms Sara Matyash for processing the polysaccharide solution prior to analysis.

References

Arad (Malis), S., Keristovsky, G., Simon, B., Barak, Z., & Geresh, S. (1993a). Biodegradation of the sulfated polysaccharide of the red microalga *Porphiridium* sp. by soil bacteria. *Phytochemistry*, *32*, 287–290.

Arad (Malis), S., Dubinsky, O., & Simon, B. (1993b). A modified cell wall mutant of the red microalga *Rhodella reticulata* resistant to the herbicide 2,6-dichlorobenzonitrile. *Journal of Phycology*, *29*, 309–313.

Basedow, A. M., & Ebert, K. H. (1977). Ultrasonic degradation of polymers in solutions. *Advanced Polymer Science*, *22*, 83–148.

Bello-Perez, L. A., Roger, P., Baud, B., & Colonna, P. (1998). Macromolecular features of starches determined by aqueous high-performance size exclusion chromatography. *Journal of Cereal Science*, *27*, 267–278.

Cohen, E., & Arad (Malis), S. (1989). A closed system for outdoor cultivation of *Porphyridium*. *Biomass*, *89*, 59–67.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Eteshola, E., Gottlieb, M., & Arad (Malis), S. (1996). Dilute solution viscosity of red microalga exopolysaccharide. *Chemical Engineering Science*, *51*, 1487–1494.

Fang, Y., Qinag, J. C., Hu, D. D., Wang, M. Z., & Cui, Y. L. (2001). Effect of urea on the conformational behavior of poly(*N*-isopropylacrylamide). *Colloid and Polymer Science*, *279*, 14–21.

Geresh, S., & Arad (Malis), S. (1991). The extracellular polysaccharide of the red microalgae: Chemistry and rheology. *Bioresource Technology*, *38*, 195–201.

Geresh, S., Lupescu, N., & Arad (Malis), S. (1992). Fractionation and partial characterization of the sulfated polysaccharide on the red microalga *Porphyridium* sp. *Phytochemistry*, *31*, 4181–4186.

Geresh, S., Mamontov, A., & Weinshtein, J. (2002). Sulfation of extracellular polysaccharides of red microalga: Preparation, characterization, properties. *Journal Biochemistry Biophysics Methods*, *50(1–2)*, 179–187.

Heaney-Kieras, J., & Chapman, D. J. (1976). Structural studies on the extracellular polysaccharide of the red alga *Porphyrydium cruentum*. *Carbohydrate Research*, *52*, 169–177.

Jones, R. F. J. (1962). Extracellular polysaccharide of the red alga *Porphyridium cruentum*. *Journal Cellular Comparative Physiology*, *60*, 61–64.

Lapasin, R., & Pricl, S. (1995). *Rheology of industrial polysaccharides: Theory and application*London: Blackie 620 pp..

Maeda, Y. Y., Watanabe, S. T., Chihara, C., & Rokutanda, M. (1988). Denaturation and renaturation of a β-1,6; 1,3-glucan, lentinan, associated with expression of T-cell-mediated responses. *Cancer Research*, *48*, 671–675.

Medcalf, D. G., Scott, J. R., Brannon, J. H., Hemerick, G. A., Cunningham, R. L., Chessen, J. H., & Shah, J. (1975). Some structural features and viscometric properties of the extracellular polysaccharide from *Porphyridium cruentum*. *Carbohydrate Research*, *44*, 87–96.

Narui, T., Takahashi, K., Kobayashi, M., & Shibata, S. (1980). A polysaccharide produced by laboratory cultivation of *Poria cocos* wolf. *Carbohydrate Research*, *87*, 161–163.

Percival, E., & Foyle, R. A. J. (1979). The extracellular polysaccharide of *Porphyridium cruentum* and *Porphyridium aerugineum*. *Carbohydrate Research*, *72*, 165–176.

Price, G. J., West, P. J., & Smith, P. F. (1994). Control of polymer structure using power ultrasound. *Ultrasonics Sonochemistry*, *1*, S51–S57.

Ramus, J. (1973). Cell surface polysaccharide of the red alga *Porphyridium*. In F. Loewus, *Biogenesis of plant cell wall polysaccharides* (pp. 333–359). New York: Academic Press.

Sato, T., Norisuye, T., & Fujita, H. (1985). Double-stranded helix of xanthan: Dissociation behavior in the mixture of water and cadoxen. *Polymer Journal*, *17*, 729–735.

Schwald, W., & Bobleter, O. (1988). Characterization of nonderivatized cellulose by gel permeation chromatography. *Journal of Applied Polymer Science*, *35*, 1937–1944.

Simon, B., Geresh, S., & Arad (Malis), S. (1993). Polysaccharide degrading activities extracted during growth of a dinoflagellate that preys on *Porphyridium* sp. *Plant Physiology and Biochemistry*, *31*, 387–393.

Yevlampieva, N. P., Pavlov, G. M., & Rjumtsev, E. I. (1999). Flow birefringence of xanthan and other polysaccharide solutions. *International Journal of Biological Macromolecules*, *26*, 295–301.

Zhang, L., Ding, Q., Zhang, P., Zhu, R., & Zhou, Y. (1997). Molecular weight and aggregation behavior in the solution of β-D-glucan from *Poria cocos* sclerotium. *Carbohydrate Research*, *303*, 193–197.