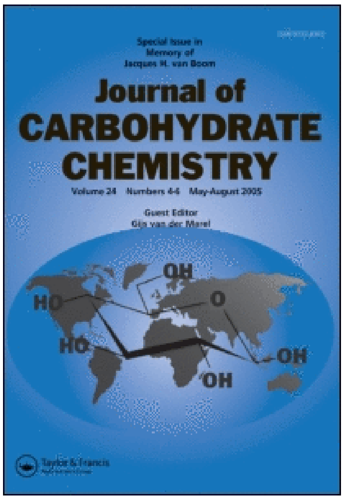

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SOLUTION PROPERTIES OF THE EXOPOLYSACCHARIDE PRODUCED BY *PSEUDOMONAS MARGINALIS* STRAIN HT041B[1]

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ABSTRACT

The solution properties of the exopolysaccharide marginalan produced by *Pseudomonas marginalis* HT041B were investigated by means of low-angle laser light-scattering, capillary viscometry, and rheology. Potentiometric and viscosimetric data indicated the absence of a cooperative transition of the disorder-to-order type. The experimental findings obtained in dilute solution (Mark-Houwink coefficients, rigidity coefficient, characteristic ratio) suggested that the polymer behaves like a semiflexible chain which adopts a disordered conformation. The rheological behaviour of more concentrated marginalan solutions, as determined by means of both steady shear and oscillatory measurements, further confirmed the disordered conformational state of the polymer in solution.

INTRODUCTION

In 1989 Osman and Fett[3] published on the isolation and the characterization of a new exopolysaccharide produced by *Pseudomonas marginalis* HT041B, as a part of a research programme focused on the characterization of exopolysaccharides produced by phytopathogenic bacteria. The polysaccharide was given the trivial name marginalan. *Pseudomonas marginalis* causes spoilage of fruits and vegetables during storage. The isolation and characterization of marginalan demonstrated that alginate is not the only acidic exopolysaccharide produced by fluorescent pseudomonads.

Marginalan was characterized as a linear galactoglucan[3,4] with the repeating unit shown in Fig. 1. Both pyruvyl and succinyl substituents are present along the polymeric chain in a molar ratio of 1:1:1:1 with the glycosidic residues. Detailed NMR investigations[4] demonstrated that both substituents are located on the galactose moiety; the pyruvyl group, which exhibits the R configuration, substituted at the 4 and 6 positions and the succinyl substituent at the 2 position.

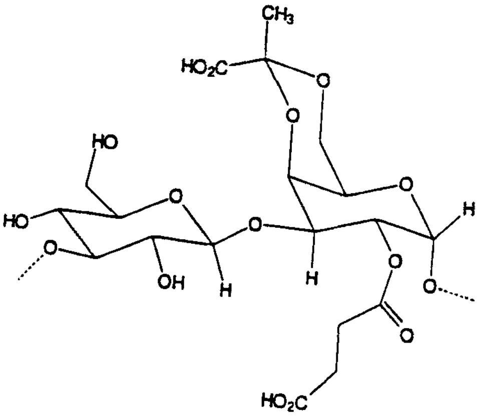
The presence of succinyl groups makes this galactoglucan different from other galactoglucans produced by *Agrobacterium radiobacter* and *Rhizobium meliloti.*[5,6,7] In fact, although the glycosidic repeating unit is identical, the latter ones contain acetyl instead of succinyl substituents, whereas the pyruvyl group is present in all the above mentioned galactoglucans.

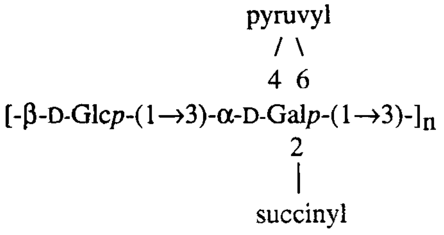
It has been suggested that marginalan might confer the proper viscosity to the bacterial micro-environment which may be an important factor for the viability of the microorganism.[8] In fact, *Pseudomonas marginalis* HT041B produces an enzyme called marginalanase[8] which is able to degrade marginalan to a lower molecular weight polymer. The presence of this enzyme strongly suggests that *Pseudomonas marginalis* might use this way to control the viscosity of the surrounding medium.

In this paper a study of the solution behaviour of marginalan is presented and the experimental findings are used to evaluate the statistical molecular properties of the polymer investigated both in perturbed and unperturbed conditions.

RESULTS AND DISCUSSION

A preliminary investigation was carried out in order to establish if marginalan is able to undergo a conformational transition of the disorder-to-order type. Due to the ionic nature of the polymer, this investigation was performed both as a function of the degree of ionization of the carboxylic groups and as a function of the ionic strength. In addition





$$\begin{array}{c} \text{pyruvyl} \\ / \ \backslash \\ 4\ \ 6 \\ [\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}]_n \\ 2 \\ | \\ \text{succinyl} \end{array}$$

Figure 1. Repeating unit of the exopolysaccharide marginalan.

to this, the interaction with divalent calcium ions was investigated by means of circular dichroism in order to determine if specific ions can induce ordered conformations.

Both the plot of the pK_a as a function of the degree of ionization α (data not shown) and the plot of the intrinsic viscosity $[\eta]$ as a function of the reciprocal of the square root of the ionic strength (NaCl) (Fig. 2) did not show any anomalous behaviour which could be related to conformational transitions. The pK_a versus α plot exhibited the normal dependence of the pK_a on the degree of ionization.[9] The plot of $[\eta]$ versus $I^{-0.5}$ showed a decidedly linear behaviour. Also, the molar ellipticity measured at 220 nm as a function of the addition of calcium ions ($Ca(ClO_4)_2$) to the polymer aqueous solution, exhibited a plateau up to a calcium to polymer ratio of 5:1.

The above findings seem to exclude the possibility that marginalan can exist in an ordered conformation. Also they might exclude the possibility of obtaining aqueous gels from marginalan solutions, since it is commonly believed that ordered conformations are the prerequisite for polysaccharide gelation.

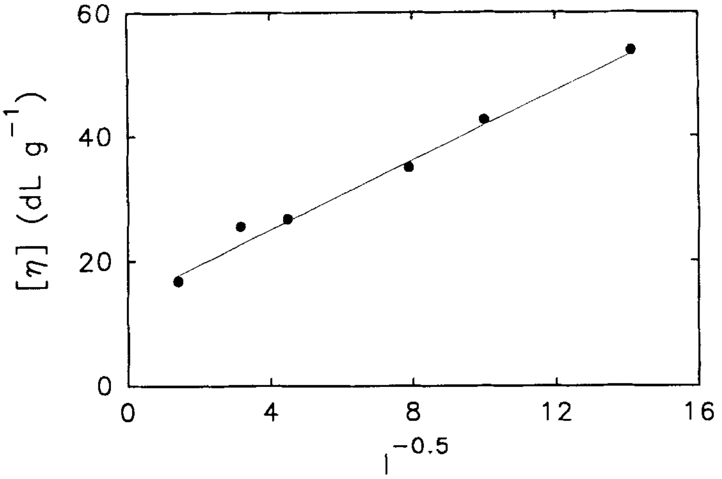


Figure 2. Dependence of marginalan intrinsic viscosity on ionic strength at 25 °C (M2 fraction).

In order to obtain the parameters related to the conformational mean properties of marginalan, six fractions exhibiting different molecular weights were obtained out of the three samples examined. All the fractions were produced by means of solvent/non-solvent fractionation except one (herein referred to as fraction MS) which was obtained by means of sonication. The intrinsic viscosity and the weight-average molecular weight of the different fractions are reported in Table 1.

The last column of Table 1 reports the sum of the Huggins and Kraemer constants as obtained from the plot of reduced viscosity as a function of the polymer concentration. As it can be seen, the value of this parameter is close to 0.5 which is the theoretical value, indicating the high accuracy of the viscosity data.

The intrinsic viscosity data, reported in Table 1, were used to obtain Mark-Houwink (M-H) equation coefficients. The double logarithmic Mark-Houwink plot is reported in Fig. 3. The experimental coefficients of the M-H equation were: a=0.65 and $K=1.22 \times 10^{-3}$ dL/g for T=25 °C. The value of the "a" constant suggests that the solvent used was a good solvent. For the sake of comparison, similar coefficients of the M-H equation have been reported in the literature for high molecular weight hyaluronic acid[10] (a=0.60; 0.15 M NaCl; $<MW_w> > 1 \times 10^{-6}$), for low molecular weight hyaluronic acid[10]

Table 1. Intrinsic viscosity ([η])at 0.1 M ionic strength, weight average molecular weight (<MW$_w$>) and the sum of the Huggins and Kraemer constants (K_H + K_K) of different marginalan fractions.

FRACTION	$[\eta]_{0.1}$ (dL g^{-1})	<MW$_w$>	$K_H + K_K$
MS	1.29	41100	0.6
F1A	4.71	530000	0.5
F1	9.40	1065000	0.5
F1B	12.79	1253000	0.4
M1	14.91	1800000	0.5
M2	26.90	5500000	0.5

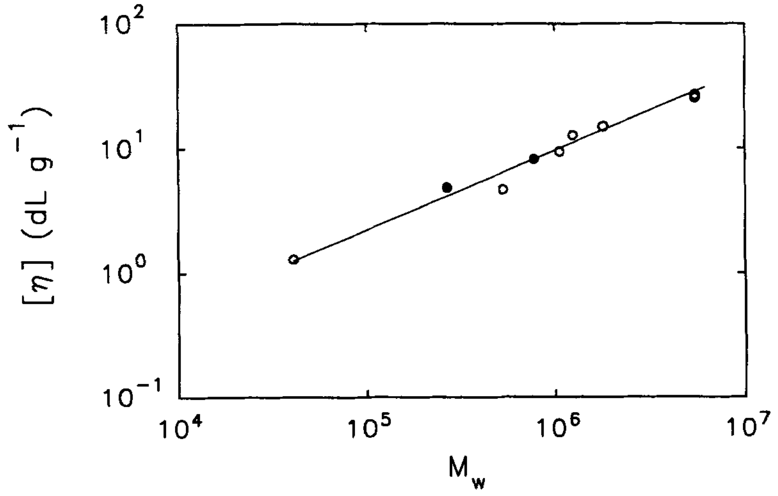


Figure 3. Mark-Houwink plot of marginalan (o) in 0.1 M NaCl at 25 °C. (•) refers to the galactoglucan produced by *Rhizobium meliloti*[13].

(a=0.78; 0.15 M NaCl; <MW_w> <1×10^{-6}), for carboxymethyl cellulose[11] (a=0.74; 0.2 M NaCl) and for guar gum[12] (a=0.723; H_2O).

Besides the intrinsic viscosity values obtained in this work, and used to define the M-H equation, Fig. 3 also shows intrinsic viscosity data obtained on the galactoglucan produced by *Rhizobium meliloti.*[13] As it can be seen, these data points interpolate the straight-line of the double logarithmic M-H plot obtained for marginalan. As already mentioned, the galactoglucan from *Rhizobium meliloti* differs from marginalan by the presence of an acetyl substituent instead of a succinyl substituent. Thus, the presence of either succinyl or acetyl substituents has the same effect on the solution conformation of the galactoglucan backbone.

Flow injection analysis (FIA) measurements[14] were carried out to obtain the weight-average molecular weights of the marginalan fractions. These measurements were also used to evaluate the second virial coefficient (A_2) which, together with both intrinsic viscosity and molecular weight, gives an indication of the flexibility and the statistical dimensions of the polymeric chain in the unperturbed state.[15] In fact, the characteristic ratio (C_x), which is connected with chain flexibility, can be obtained by the following equation:

$$C_x = \left(\frac{[\eta]}{\phi \langle M_v \rangle^{\frac{1}{2}}} \right)^{\frac{2}{3}} \left(\frac{M_u}{\alpha^2 L^2} \right)$$

where ϕ is the Flory-Fox parameter (2.2×10^{23} mol^{-1}), L is the length of the virtual bond relative to one saccharidic unit (0.415 nm for marginalan), <M_v> is the viscosimetric mean molecular weight, and α^2 is the so-called expansion factor.

For our calculations, the viscosimetric mean molecular weight was derived from the weight-average molecular weight by means of the following equation:[16]

$$\langle M_v \rangle^{\frac{1}{2}} = 0.94 \langle M_w \rangle^{\frac{1}{2}}$$

where <M_w> values were obtained by means of gel permeation chromatography coupled with low angle laser light scattering detection.

The expansion factor has been obtained according to the procedure reported in the literature:[15,16]

$$\alpha^2 = 1 + 0.233[\exp(5.73\varphi) - 1]$$

$$\varphi = \left(4\pi^{\frac{3}{2}}N\right)^{-1} A_2 \langle M_w \rangle^{\frac{1}{2}} \left(\frac{6^{\frac{3}{2}} \phi \langle M_v \rangle^{\frac{1}{2}}}{[\eta]} \right)$$

where N is Avogadro's number and A_2 is the second virial coefficient.

Furthermore, the value of the gyration radius (R_g) can be easily obtained from the definition of the characteristic ratio provided that the relation which includes the mean end-to-end distance ($\langle r^2 \rangle = 6\ R_g^2$) is used. In fact, $\langle r^2 \rangle = C_x \langle n \rangle L^2$ where <n> is the mean number of residues of length L in the polymeric chain.

The mean conformational parameters obtained for marginalan using the above equations are reported in Table 2.

On one hand, as expected, both the mean end-to-end distance and the gyration radius increase as a function of increasing molecular weight. On the other hand, the characteristic ratio value is fairly constant, apart for the figure relative to the lowest molecular weight fraction for which probably the equations used become less reliable. The plateau figure of C_x can then be considered as representative of the C_∞ value. In addition to this, the constancy of the C_x parameter over different fractions is a good check for the similarity of the molecular weight distribution curves relative to the polymeric fractions investigated. It is worth noting that a characteristic ratio equal to 20 was obtained for the (1→3)-β-Glc*p*-(1→3)-α-Gal*p* polymer by means of theoretical conformational analysis[13]. In addition to this, the figures reported in Table 2 are in agreement with the behaviour of a semi-flexible polymer.

The unperturbed value of the characteristic ratio can also be obtained either using the Stockmayer-Fixman equation or by means of the Hearst treatment[17]. Both the methods are aimed at the evaluation of the K Mark-Houwink constant in the unperturbed state (K_θ) trying to overcome the difficulty of carrying out experiments under conditions that are particularly difficult for polysaccharidic systems. The unperturbed K_θ M-H constant can then be correlated to the C_∞ characteristic ratio using the equation

$$K_\theta = \phi L^3 \left(\frac{C_\infty}{M_u} \right)^{\frac{3}{2}}$$

where the symbols have the above reported meaning and M_u is the molecular weight of the glycosidic unit (M_u=269).

Table 2. Second virial coefficient (A_2), expansion factor (α^2), characteristic ratio (C_x), end-to-end mean distance ($<r_x^2>^{1/2}$) and gyration radius (R_g) for different marginalan fractions.

FRACTION	F1A	F1	F1B	M1	M2
A_2 (cm^3 mol g^{-2})	$6.8 \cdot 10^{-4}$	$8.8 \cdot 10^{-4}$	$12.5 \cdot 10^{-4}$	$9.9 \cdot 10^{-4}$	$5.8 \cdot 10^{-4}$
α^2	1.38	1.60	1.88	1.84	1.83
C_x	25.1	27.2	27.0	27.0	27.7
$<r_x^2>^{1/2}$ (nm)	92.3	136.2	147.0	176.5	312.1
R_g (nm)	37.7	55.6	60.0	72.1	127.4

The Stockmayer-Fixman plot is reported in Fig. 4. The C_∞ value obtained from this plot was 31.1, rather different from the figures which were obtained for the C_x parameter reported in Table 2. A value (C_∞= 29.6) more similar to those reported in Table 2 was obtained from the Hearst plot (Fig. 5). The above findings are not surprising since the Hearst equation was specifically devised for semiflexible polymers, as marginalan seems to be. In addition, the better linearity of the Hearst plot further confirms the semiflexible nature of marginalan.

The conformational behaviour of marginalan depends on the ionic strength of the solvent, as marginalan is a polyelectrolyte. In the experiments described above the ionic strength was always 0.1 M. It is useful, however, to derive a parameter which takes into account the dependence of the intrinsic viscosity on the ionic strength. Smidsrød and Haug[18] derived a simple parameter (B) from the slope (S) of the plot of [η] as a function of the inverse of the square root of the ionic strength (see Fig. 2 for marginalan).

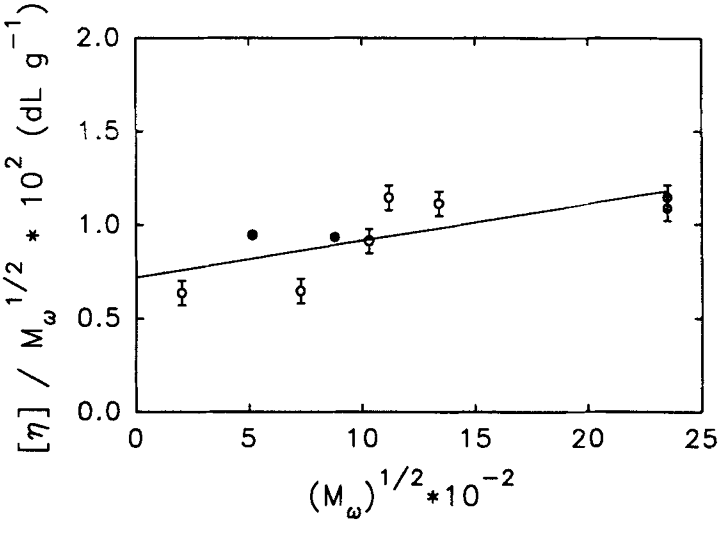


Figure 4. Stockmayer-Fixman plot of marginalan (○) in 0.1 M NaCl at 25 °C. (●) refers to the galactoglucan produced by *Rhizobium meliloti.*[13]

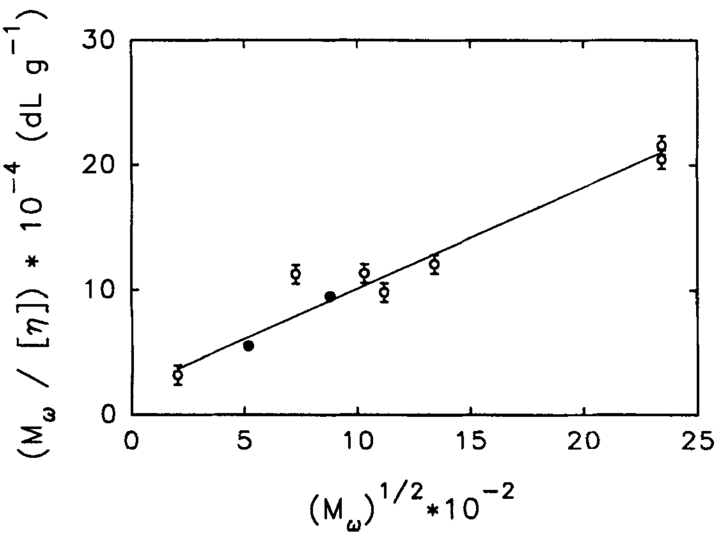


Figure 5. Hearst plot of marginalan (○) in 0.1 M NaCl at 25 °C. (●) refers to the galactoglucan produced by *Rhizobium meliloti.*[13]

Moreover, a specific value of the intrinsic viscosity ([η] at 0.1 M ionic strength) was taken into account as a reference state:

$$B = \frac{S}{[\eta]_{0.1}^{v}}$$

where v assumes values in the range 1.2-1.4.

The B parameter is proportional to a number of entities linked to the flexibility of the polymeric chain, and, in addition to this, can be conveniently used to compare the conformational behaviour of different polyelectrolytes. The B value obtained for marginalan was 0.049 which falls in the typical range of semiflexible polymers like hyaluronate (B=0.065)[18] and mannuronic-rich alginate (B=0.04).[18]

The characterization of the rheological properties of marginalan (fraction M2) was also performed in the finite concentration regime by means of a rotational rheometer operated both in the steady-state and in the oscillatory mode. Fig. 6 shows the shear rate dependence of viscosity for marginalan at different polymer concentrations.

The rheological behaviour of marginalan is of the pseudoplastic type. As shown in Fig. 6, a "Newtonian plateau" can still be recognized at the highest polymer concentrations used, and a value of the steady shear viscosity at zero shear, η_0, can then be established.

It is useful to report the η_0 findings together with the specific viscosity data, as obtained by means of capillary viscosimetry, in a master plot where the specific viscosity is reported as a function of the product c[η], which is called the coil-overlap parameter and where c is the polymer concentration. This plot is shown in Fig. 7 for marginalan fraction M. Two different behaviours characterised by different slopes were detected. Typically, these two domains define two different regimes: the dilute one, obtained at low values of the coil-overlap parameter, and the semi-dilute regime at higher coil-overlap values. For marginalan, the point at c*[η]=2.4 indicates the critical polymer concentration (c*=0.09 %) where polymer-chain overlapping starts to be dominant, therefore passing from the first regime to the second one.

The rheological behaviour of marginalan is typical of solutions of polysaccharides which adopt a disordered conformation; here again, the critical coil-overlap parameter (2.4) is very similar to that obtained for semiflexible polymers like hyaluronate (c*[η]=2.5).

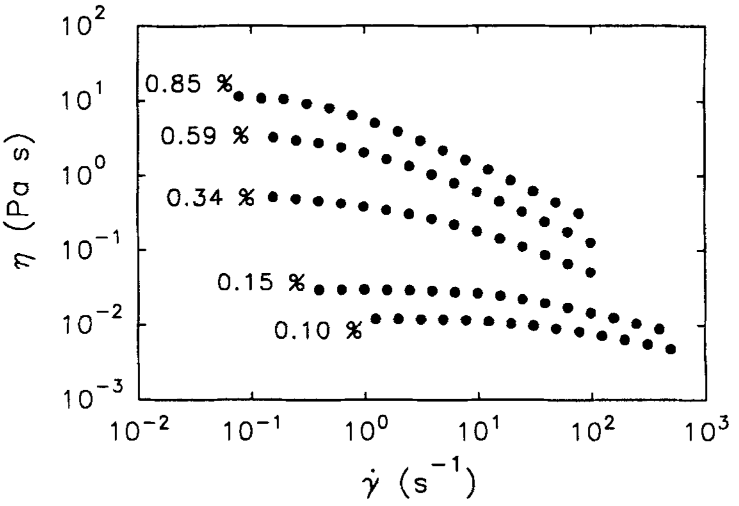


Figure 6. Shear rate dependence of viscosity for marginalan (fraction M2) in 0.1 M NaCl at 25 °C. Experiments using different polymer concentrations (Cp= % w/v) are reported.

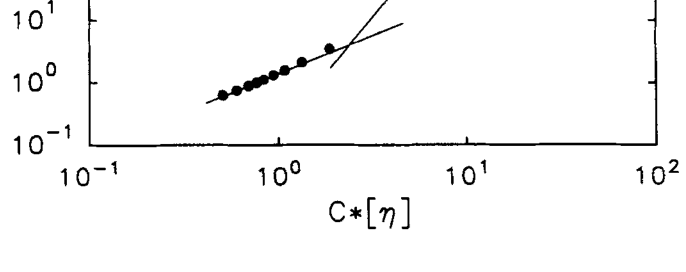


Figure 7. Variation in polymer solution viscosity as a function of the coil-overlap parameter for marginalan (fraction M2) in 0.1 M NaCl at 25 °C.

In addition to this, the values of the two slopes shown in Fig. 7 (1.3 for the dilute regime and 3.6 for the semidilute one) are in good agreement both with the theory[19] and with findings relative to other polysaccharides which adopt a disordered conformation (guar gum, locust bean gum, and hyaluronate[20]).

Rheological measurements carried out in the oscillatory mode as a function of the oscillatory frequency made it possible to determine two parameters which are particularly relevant for the characterization of aqueous polysaccharide systems: the elastic modulus and the loss modulus. In fact, the prevalence of one modulus over the second one is used for the definition of either "liquid-like" systems (loss modulus larger than the elastic one) or "gel-like" systems (elastic modulus larger than the loss one). The inversion point, where the system passes from the liquid-like to the gel-like regime, defines the crossover oscillation frequency.

A typical plot reporting the two modulii as a function of the rheometer oscillation frequency for marginalan fraction M2 (polymer concentration = 0.85 % w/v) is shown in Fig. 8, where the crossover frequency is clearly shown.

Typically, the crossover frequency shifts to higher oscillation frequencies when lowering the polymer concentration (data not shown).

The value of the complex viscosity η^* is also reported in Fig. 8. This parameter, which is given by an algebraic combination of both the elastic and the loss modulus,[17] can be used to further characterize the conformational state of the polymer investigated. In fact, the graph obtained by reporting on the same plot both the complex viscosity as a function of the oscillation frequency, and the steady shear viscosity as a function of the shear rate, clearly shows that the parameters superimpose very nicely (Fig. 9).

This behaviour is known as the Cox-Merz rule[21] and is valid for polymeric solutions where the polymer adopts a disordered conformation. Contrary to this, polysaccharides which assume ordered conformations do not follow the Cox-Merz rule and, in these cases, the complex viscosity is larger than the steady shear viscosity.

EXPERIMENTAL

Materials. Solutions of marginalan were prepared by dissolving known weights of the polysaccharide in the proper buffer solutions with stirring for at least 3 h at room

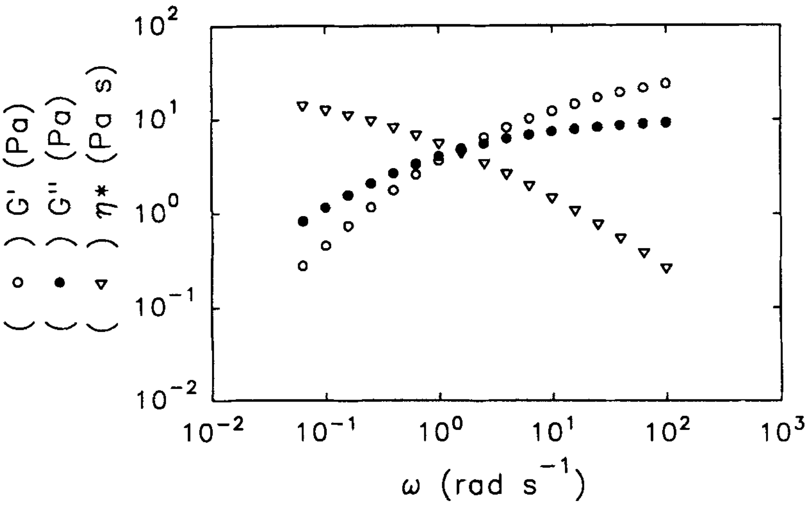


Figure 8. Viscoelastic spectra of marginalan (fraction M2, Cp=0.85 % w/v) in 0.1 M NaCl at 25 °C. The frequency dependence of the complex viscosity (η*), the loss modulus (G"), and the elastic modulus (G') is reported.

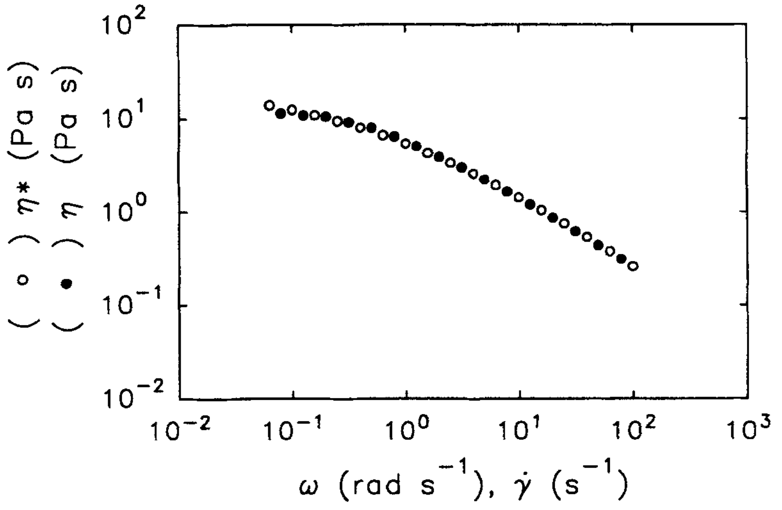


Figure 9. Dependence of both the complex viscosity and the steady shear viscosity for marginalan on oscillation frequency and shear rate (fraction M2, Cp=0.85 % w/v) in 0.1 M NaCl at 25 °C.

temperature. Concentrations were corrected for the water content of the lyophilised samples (Karl-Fischer titration).

Fractionation. Two marginalan fractions (hereafter called M1, M2) characterized by different molecular weights have been obtained from the fermentation broths at the USDA. A third marginalan fraction (referred as M0 and obtained from the bacteria culture) was used together with the M1 fraction to obtain different molecular weight marginalan fractions.

A mixture of M0 and M1 was stirred in a 1:1 water-isopropyl alcohol solution for several hours in order to allow for the partial solubilization of the polysaccharide. The suspension was centrifuged (20,000 rpm, 20 minutes) and the resultant supernatant fluid was dried on a rotary evaporator. The insoluble material was suspended again in 1:1 water-isopropyl alcohol and was stirred overnight. The suspension was centrifuged (20,000 rpm, 30 minutes) and the supernatant fluid was added to the previously obtained residue and then dried on a rotary evaporator, solubilized in distilled water and finally freeze-dried. This material was designed as the F1 fraction.

A further fractionation of the F1 fraction was accomplished by adding isopropyl alcohol dropwise to an aqueous marginalan solution (0.5 g/L) until the solution became turbid (about 3 volumes). After centrifugation the supernatant was dried on a rotary evaporator and the residue obtained was solubilized in water and freeze-dried. This material was designed as the F1A fraction. The pellet was dissolved in distilled water, dried on a rotary evaporator and then re-solubilized in distilled water and lyophilized to give the F1B fraction.

Another fraction (called MS) was obtained from a mixture of M1, M2, F1, F1A and F1B samples by means of sonication with a MSE-Soniprep 150 Ultrasonic Disintegrator. The treatment was carried out on 50 mL of an aqueous polysaccharide solution (2 g/L) cooled in an ice-water bath by sonicating at maximum power (150 W) for a total of 4.5 h. Sonication was done at intervals of 6 min with 3 min cooling periods. Finally, the solution was filtered (5 μm Millipore filter) and the filtrate freeze-dried.

Capillary Viscometry. Viscosity measurements were carried out by means of a Schott-Geräte automatic viscometer equipped with an Ubbelohde capillary (0.46 mm), immersed in a Lauda thermostat bath, and an AVS 440 measuring system.

Before each measurement the polymer solution was filtered through a 1.2 μm Millipore filter, whereas the solvent was filtered through a 0.45 μm Millipore filter.

Measurements carried out as a function of ionic strength were performed at 25 °C on solutions dialysed overnight against various concentrations of aqueous NaCl. The dialysis outer solutions were then used as dilution solvent in the determination of the intrinsic viscosity. Intrinsic viscosity was determined by the double extrapolation of both the Huggins and the Kraemer plot.

Rheology. Experiments were performed by means of a Rheometrics RFS 8500 instrument equipped with a cone and plate geometry (50 mm diameter, 0.04 radiant cone angles). This kind of geometry has the advantage of providing a homogeneous shear rate throughout the sample. Both steady shear measurements, in the range 5×10^{-2} to 5×10^{2} s^{-1}, and dynamic oscillatory measurements, in the range 5×10^{-2} to 10^{2} rad s^{-1}, were carried out on concentrated marginalan solutions in 0.1 M NaCl at 25 °C.

Light Scattering. Low angle light-scattering measurements, for the determination of the weight-average molecular weight, $<MW_w>$, and the second virial coefficient, A_2, were carried out by means of a Chromatix TSP/KMX-6 laser light-scattering instrument on polysaccharide solutions in 0.1 M NaCl at 25 °C. For each sample, solutions at different polymer concentrations were obtained by diluting a concentrated solution and filtering through a 0.45 μm Millipore filter. The resulting solutions were then injected in the instrument at a flow of 0.5 mL/min through a 0.45 μm cellulose filter. The differential refractive index increment, (dn/dc), was determined by means of a Chromatix KMX-16 laser differential refractometer and resulted to be 0.1585 mL/g.

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2. Reference to brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

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