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Molecular Weight of Scleroglucan and other Extracellular Microbial Polysaccharides by Size-exclusion Chromatography and Low Angle Laser Light Scattering

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SUMMARY

High-performance aqueous size-exclusion chromatography coupled to a low angle laser light scattering detector has been applied to the analysis of scleroglucan and various other extracellular microbial polysaccharides. Emphasis has been focused on three main findings. (1) The molecular weight of these macromolecules is not very sensitive to changes in fermentation conditions. This is specially true in the case of scleroglucan and related (1→3)-β-D-glucans including schizophyllan, which all exhibited a constant weight-average molecular weight of $5{\cdot}7\times10^6 \pm 5\%$. *(2) In contrast to plant polysaccharides, polydispersity is very low, usually near unity. (3) The molecular weight levels off quickly during biosynthesis since the molecular weight is constant from the middle of fermentation, if not before.*

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INTRODUCTION

Microbial polysaccharides represent a very promising potential source of new polysaccharides (Sturgeon, 1983). At present xanthan, by its industrial tonnage, largely surpasses the other microbial gums; however, some polysaccharides, like scleroglucan commercialized by the French company Satia, are under development. Scleroglucan belongs to a class of structurally related polysaccharides produced by various species of plant pathogen fungi, especially those of genus *Sclerotium*. The backbone consists of (1→3)-linked β-D-glucopyranosyl residues, with side chains of single, (1→6)-linked β-D-glucopyranosyl residues for every third glucose in the main chain (see Fig. 1). This structure has been first elucidated by periodic oxidation and hydrolysis with selected enzymes (Johnson *et al.*, 1963) and recently confirmed by methylated sugar analysis (Heyraud & Salemis, 1982) and by ^{13}C-NMR studies (Rinaudo & Vincendon, 1982). Scleroglucan is produced industrially in conventional fermenters by culture of selected strains of *Sclerotium rolfsii* in a liquid nutritional medium (Halleck, 1967). This medium contains glucose, a nitrogen source and selected minerals.

Scleroglucan is generally used in aqueous solution where macromolecules are self-associated in a triple helix arrangement. This struc-

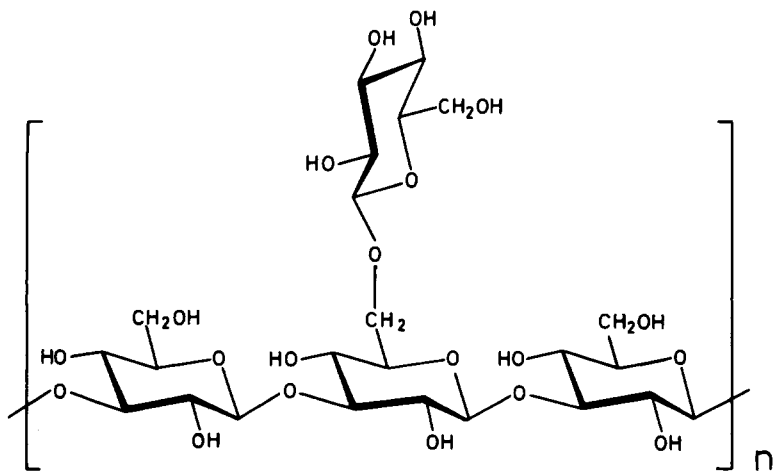

Fig. 1. Repeat unit of scleroglucan.

ture confers to macromolecules a rigid rod-like structure (Bluhm *et al.*, 1982; Yanaki & Norisuye, 1983) in which the branched glucose units are on the outside of helices and thus prevent higher aggregates. The triple helix structure may exist over a wide range of conditions but chains are dispersed as random coils when the pH value is greater than 12·5 (Bluhm *et al.*, 1982), or when dissolved in DMSO (Yanaki *et al.*, 1981).

Functional properties of scleroglucan solutions are due to the rigid, rod-like structure. The main properties of concentrated solutions are a temperature-stable high viscosity and a pseudoplastic behaviour with a high yield value, which gives them extremely useful suspending properties (Brigand, 1986). Dilute solutions are exceptionally viscous. Additionally, they present outstanding shear and chemical stability, compatibility with the usual chemicals and electrolytes, and good filterability. Such properties make scleroglucan very interesting for numerous applications in food, industrial or pharmaceutical areas. But the most promising outlet is the oil industry, where scleroglucan can be used for thickening drilling muds, hydraulic fracturing (Desbrières, 1983) and completion fluids, but mainly for enhanced oil recovery (Williams, 1968; Akstinat, 1980; Teeuw & Hesslink, 1980). In the last case, scleroglucan is particularly valuable for use under conditions of high temperature and salinity (Davison & Mentzer, 1982), where it performs more efficiently than those of xanthan and where polyacrylamides are not used because of their lack of stability and compatibility. Elf Aquitaine is currently funding an important effort to develop the use of scleroglucan for enhanced oil recovery. Recent progress in the manufacturing process and enhancing the functional characteristics of the biopolymer have been presented at the Biotech '85 Conference (Donche, 1985).

Only two laboratories have published data on molecular weight and molecular weight distribution of scleroglucan. A Japanese team from Osaka University has studied it by light scattering. The weight-average molecular weight of the native sample in the triple helix form is $5{\cdot}4 \times 10^6$ (Yanaki *et al.*, 1981), and various properties appear similar to that of schizophyllan with $M_w = 5{\cdot}7 \times 10^6$ (Norisuye *et al.*, 1980).

Another method, coupling of size-exclusion chromatography (SEC) and low angle laser light scattering (LALLS) in DMSO, has been described (Salemis & Rinaudo, 1984). The molecular weight is 10 times lower ($M_w = 540\,000$ and $M_n = 231\,000$), when only a three-fold

reduction would be expected. Such a disagreement may be easily rationalized by classical difficulties in the analysis of high molecular weight polysaccharides. For example, in the case of xanthan, absolute molecular weight was a matter of heated debate (Launay *et al.*, 1984). Because of these difficulties, significant effort has been expended in the Elf Aquitaine research programme to develop the coupled SEC–LALLS for the scleroglucan analysis, to control the potential effect of synthetic parameters on the molecular weight distribution and to make valid and necessary comparisons with other extracellular microbial polysaccharides.

EXPERIMENTAL

Samples

Most of the samples that have been analysed are listed in Table 1. Identity, origin and some remarks are given with solution viscosity at room temperature. Brookfield viscosities at 6 rpm ($7{\cdot}35\ s^{-1}$) were measured on 750 ppm solutions in 0·5 M NaCl. A UL adapter was used with the LVT model. Possible difficulties in the solubilization are also specified.

Scleroglucan samples A–G were products from Satia, with differences either in the fermentation process or in the various steps of

TABLE 1
List of Main Samples

Nature of polysaccharide	*Sample*	*Origin*	*Observations*	*Viscosity (cP)*	*Difficulty in solubilization*
Scleroglucan	A	Satia	Typical products with	52	
	B	Satia	various changes in	50	
	C	Satia	recovery of powder	50	+
	D	Satia		48	+
	E	Satia	Dilution of two native	48	
	F	Satia	broths	50	
	G	Satia	Mineral nitrogen source	48	
	H	Satia	First products	16	
	I	Satia	obtained at Satia in the beginning	18	

Table 1 — *contd.*

Nature of polysaccharide	*Sample*	*Origin*	*Observations*	*Viscosity (cP)*	*Difficulty in solubilization*
	J	Satia	Samples taken during a fermentation: J 11·5 h, K 21·5 h and L 45 h	—	+ + +
	K	Satia		—	+
	L	Satia		—	
Related glucans	M	EBR	*Sclerotium rolfsii* ATCC 15205	40	
	N	EBR	*Sclerotium delphinii* ATCC 15197	42	+
	O	EBR	*Sclerotium rolfsii* ATCC 16648	36	+ +
	P	EBR	*Corticium rolfsii* ATCC 15210	44	
	Q	EBR	*Sclerotium rolfsii* ATCC 24459	45	
	R	EBR	*Sclerotium rolfsii* ATCC 26326	47	
Schizophyllan	S	Japan			+ +
Xanthan	X1	Kelco		24	
	X2	Rhone-Poulenc	Grinding to 40 μm	12	
	X3			25	
	X4	Shell		—	
	X5	Satia	Products obtained with various changes including nitrogen source, temperature of sterilization and fermenter volume	11	
	X6	Satia		34	
	X7	Satia		26	
	X8	Satia		42	
	X9	Satia		—	
	X10	Satia		32	
	X11	Satia		13	
	X12	Satia	10 h Samples taken during a fermentation (X17 is the final product)	—	
	X13	Satia	20 h	—	
	X14	Satia	30 h	—	
	X15	Satia	40 h	—	
	X16	Satia	50 h	—	
	X17	Satia	60 h	—	
Pseudomonas elodea polysaccharide	G1	Kelco	Gellan gum	< 5	
	G2	Kelco	Gellan gum	< 5	
	G3	EBR		< 5	Gel at 20°C
	G4	EBR	Deacetylated form of G3	< 5	
New polysaccharide	T	Satia		69	

powder recovery. H and I were two unusual samples, obtained at the beginning of scleroglucan development at Satia. J, K and L were samples taken during the course of fermentation (at 11·5, 21·5 and 45 h, respectively, for a total duration of 60 h). M to R were produced at EBR (Elf Bio Recherches, Labege, 31328 Castanet Tolosan, France) from various strains; some are described in the original patent (Halleck, 1967). They are not scleroglucan, but of similar structure.

Similarly, xanthans X5 to X11 were obtained after various changes in the process (nutritional medium, sterilization conditions, etc.) and X12 to X16 were samples taken during a fermentation for which the final product was X17. A few analyses were made on *Pseudomonas elodea* polysaccharides. Finally, a further two samples were examined: a new polysaccharide T in the course of development at Satia, and a native schizophyllan (sample S), kindly provided by Professor M. Rinaudo.

Chromatographic apparatus

The high-pressure liquid chromatograph consisted of the following parts: a Waters 6000 A solvent delivery system, a Waters U6K injector and a Jobin-Yvon Iota differential refractometer. A set of two columns (Toyosoda, TSK PW type, each 60 cm in length) was used, either PW 6000 + PW 4000 or PW 6000 + PW 5000, interchangeably. The whole system was thermostatted with a Lauda electronically controlled water bath. A Chromatix CMX 100 LALLS detector was inserted on-line between the columns and the refractometer. It was equipped with its own high temperature accessory. A home-made filter housing was connected in front of the cell inlet. Filters of adequate diameter were taken from Millipore SSWP 04700 membranes, with 3 μm pore size. Water used was taken from a Milli-ro + Milli-q water purification system from Millipore, and was carefully degassed and filtered through Millipore 0·22 μm GS membranes before use.

Refractive index increment

A Chromatix KMX-16 differential refractometer operating with the He/Ne 632·8 nm laser light (the same as in the CMX-100) was used to determine dn/dc values in the conditions selected for SEC analysis (where n is the refractive index of the solution and c is the concentration of polymer in the solvent).

RESULTS

Choice of SEC conditions

Recent results show that the SEC analysis of scleroglucan may be performed under mild conditions, i.e. 0·1 M $NaNO_3$ as eluent, at room temperature. However, for ease of comparison, we maintained constant conditions for the great bulk of plant or microbial polysaccharides we were concerned with, including scleroglucan. Conditions that were developed for carrageenans (Lecacheux *et al.*, 1985), i.e. Toyosoda PW columns and 0·1 M LiCl as eluent at 60°C, are drastic enough to permit elution of gel forming products like agar and gellan. Such a system has appeared very convenient for scleroglucan too, as will be clear in this report. The chromatogram of sample A (see Fig. 2) is a typical example of routine analysis. Concentration was 500 ppm and injected volume 100 μl which yielded a molecular weight of several million is obtained, with no dependence on injected quantity.

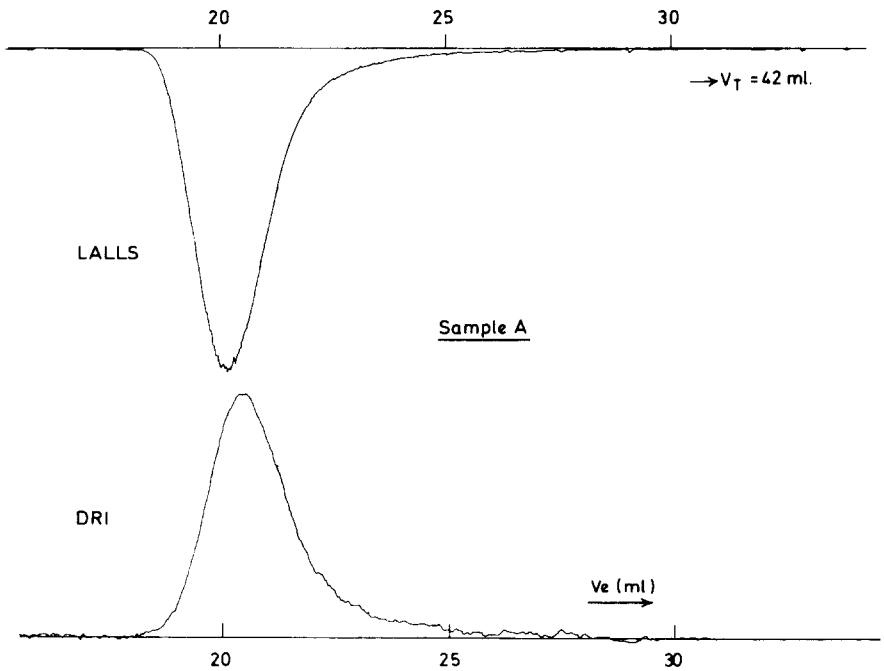

Fig. 2. Chromatograms of sample A (injected quantity 50 μg).

As mentioned in the introduction, it is well established that scleroglucan and other similar (1→3)-β-D-glucans dissolve in aqueous solvents as rod-like trimers or higher aggregates; single randomly coiled chains are formed beyond pH 12·5 or in organic solvents like DMSO. So, it was attractive to compare results from our classical conditions with chromatograms in other solvents. Since DMSO was excluded, due to its lack of similarity to water, the usual application solvent, a trial was made with 0·2 M NaOH at room temperature. Unfortunately, degradation obviously occurred as well as triple helix melting, and the experiment was stopped. On the contrary, SEC in 0·05 M NaOH, as recently reported (Itou & Teramoto, 1984) in the case of schizophyllan, was a very positive test, because the results were exactly the same as that with 0·1 M LiCl as eluent, which (a) confirms the validity of our classical SEC conditions and (b) eliminates the possibility that higher aggregates may co-exist with triple helix rods.

Preparation and filtration of solutions

Solutions were made routinely at 0·5 g litre^{-1} (500 ppm) directly in the SEC eluent (0·1 M LiCl). Roughly 20 ml were prepared in a flask immersed in a water bath at 60°C using magnetic stirring. If the solution remained cloudy after 3–4 h (see Table 1), powder was dissolved in 10^{-2} M NaOH under similar conditions. A comparison of the SEC chromatograms was made on sample A, which was very soluble in pure water: solubilization at pH 12 did not affect the molecular weight. Some samples were solutions ranging from 1 to 30 g litre^{-1}. A simple dilution was made to adequate concentration, without any previous purification.

Filtration was made on Millipore membranes. Significant retention was observed on porosities that are commonly used for analytical purposes, and very reproducible data were obtained by monitoring the area of the DRI response: 73% retention on 0·22 μm, 24% on 0·45 μm and 10% on 0·8 μm. Fortunately, no loss was observed on 3 μm SSWP 04700, which was finally chosen for use.

These results are not surprising. According to reported data from the Japanese researchers on scleroglucan as well as schizophyllan (Yanaki *et al.*, 1980; Kashiwagi *et al.*, 1981; Norisuye, 1985), the equivalent volume of the rigid triple helix is very large, since the radius of gyration is found to be 0·3 μm, and persistence length is found to be 0·2 μm. Loss on porosities less than 1 μm is thus obviously normal.

Refractive index increment

Xanthan X11 and scleroglucan sample A were subjected to refractive index increment measurement in the SEC eluent at 60°C. For the xanthan gum, the measurement was perfect at the first attempt, and in concordance with previously measured data. The value of 0·141 was obtained. On the contrary, it was very difficult to obtain a reasonable fit on data for scleroglucan. The reasons were probably the relative heterogeneity of the moderately concentrated solutions (2–3 g litre^{-1}) and loss on filtering. By chance, we observed that the DRI response was the same, both for xanthan and scleroglucan, within 1% confidence limits. A dn/dc of 0·14 was thus assumed for scleroglucan, which gives $M_w = 5{\cdot}7 \times 10^6$ for the SEC result on sample A. Other microbial polysaccharides have not been submitted to this measurement. Molecular weight results have been computed with the assumption that 0·14 would be equally correct.

Second virial coefficient measurement

It is well accepted that the value of A_2 may have an important effect on the validity of the SEC–LALLS results (Martin, 1982). This is why an attempt was made to determine the second virial coefficient on sample A, using the LALLS detector in an almost static mode. Columns were removed and 2·5 ml of solutions with increasing concentrations were successively analysed at a low flow rate (0·5 ml min^{-1}). Despite numerous spikes, a good correlation was obtained, as can be seen from Fig. 3.

Classical regression analysis leads to $A_2 = -1{\cdot}6 \times 10^{-3}$ mol ml g^{-2} and $M_w = 6{\cdot}0 \pm 0{\cdot}5 \times 10^6$ g mol^{-1}. Since the weight-average molecular weight is in good agreement with that deduced from the SEC–LALLS coupling, the value of A_2 is very surprising. Such a value for A_2 would have a dramatic effect on the SEC result, making it a function of the injected quantity, even though our experimental observations suggest the opposite view.

Figure 4 is the plot of the LALLS signal area versus the injected quantity. It is obvious that the linearity is good, with a correlation factor of 0·994. This graph is the best proof that A_2 does not play any significant role in the SEC result. Mathematical examination of the experimental points may indicate a slight downward curvature, and thus a positive value of A_2. An estimate has been deduced from

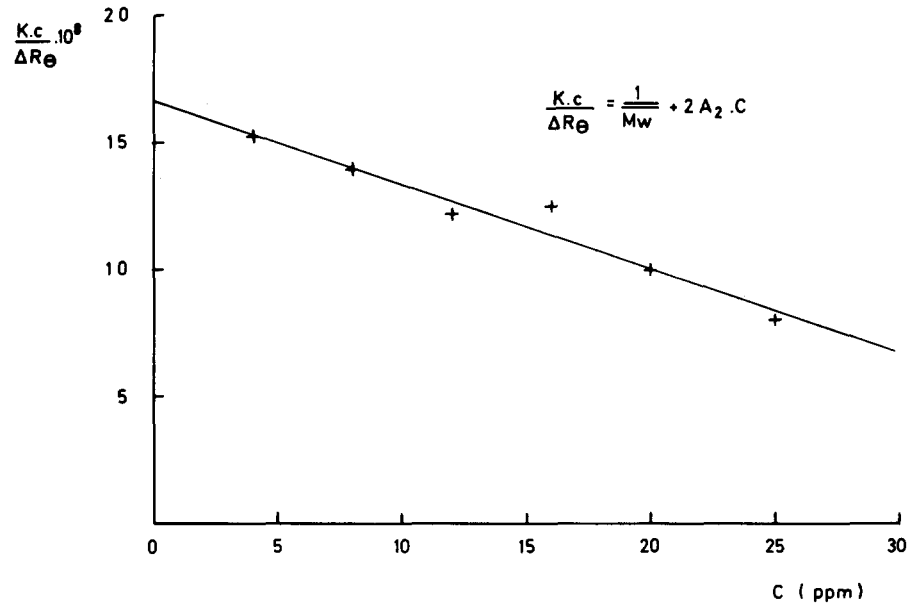

Fig. 3. Static light scattering experiment on sample A.

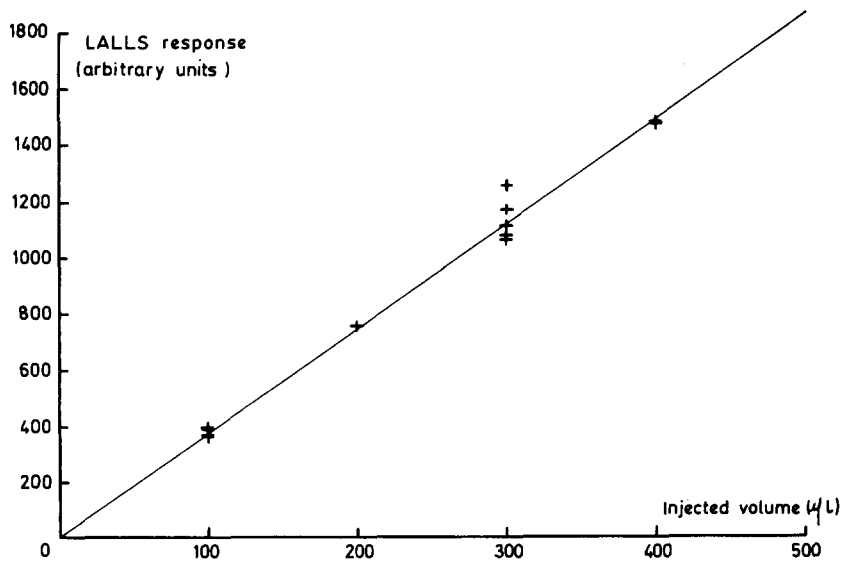

Fig. 4. Dependence of LALLS signal area with injected quantity.

second-degree polynominal regression of $A_2 = 5 \times 10^{-5}$ mol ml g^{-2}, a value in concordance with published results (Yanaki *et al.*, 1981). Discrepancy between the two methods is probably a consequence of the tendency of self-association, which of course varies with shear rate and flow conditions.

Similarly, xanthan X11 gave a straight line as in Fig. 4. It was thus reasonable to neglect the A_2 contribution for all results presented in this report.

Various pitfalls in the search for absolute results

Before the presentation of absolute molecular weights, it is important to list all the pitfalls that could invalidate these SEC–LALLS results.

It has been previously reported (Lecacheux *et al.*, 1985) that high-performance SEC of high molecular weight polysaccharides is performed under non-equilibrium conditions. Thus, if the weight-average molecular weight is not affected, polydispersity I is erroneous. It is clear that I values in this paper are the correct order of magnitude for comparison but not for absolute results, especially as macromolecules are eluting near the void volume. Diffusion broadening of peaks has not been corrected for. Moreover, concentration effects are present, and a very slight adsorption is even sometimes observed. Possible effects from shear degradation are negligible since molecular weights are constant over a range of flow rates, including the almost static experiments.

Occurrence of numerous spikes on the LALLS response may result in dramatically overestimated molecular weights, showing the importance of changing the pre-LALLS filter. On the contrary, providing that the base line is settled, its absolute position does not affect the M_w value, as long as the parasitic light does not exceed by a factor of 3–5 the Rayleigh scattering component of the eluent.

Molecular weight of scleroglucan

We can now summarize all the results. For scleroglucan sample A, we obtained $M_w = 5{\cdot}7 \times 10^6 \pm 5\%$, $I < 1{\cdot}2$. Figure 5 represents the apparent molecular weight distribution, with some scatter due to the narrowness of the distribution and to the vicinity of the void volume.

With a few exceptions that will be given below, all scleroglucan samples (see Table 1) were very similar in molecular weight. What is

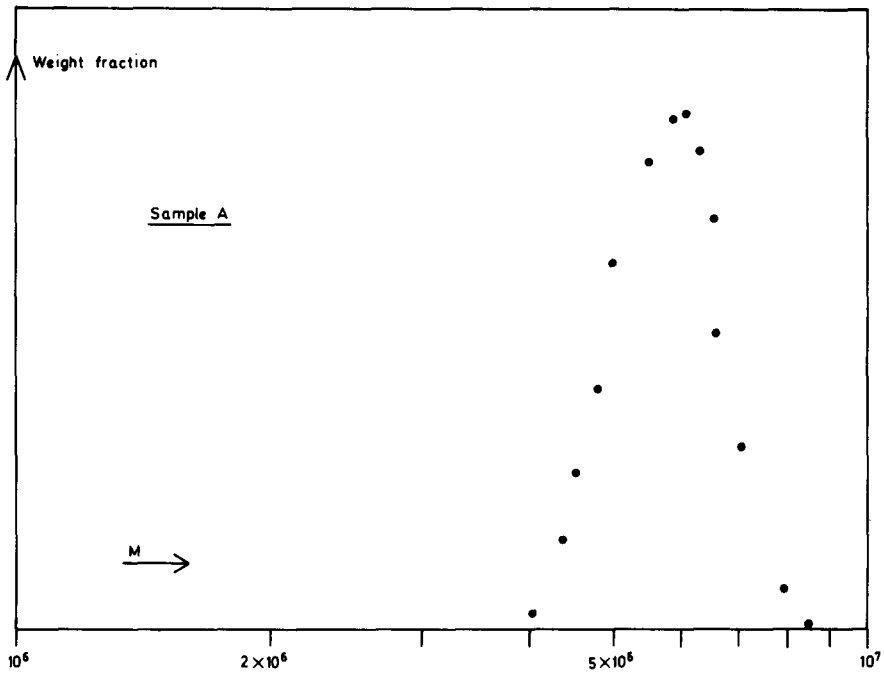

Fig. 5. Molecular weight distribution of sample A.

more, related glucans M to R obey the same rule, with $M_w = 5{\cdot}7 \times 10^6 \pm 5\%$. Schizophyllan sample S was identical within experimental error, which completes the agreement of our results with those of Yanaki (Yanaki *et al.*, 1980).

The exceptions were sample J, which had low solubility even in 10^{-2} M NaOH and did not allow an accurate measurement, and samples H and I whose degradation history was not known: $M_w = 2{\cdot}7 \times 10^6$ and $I = 1{\cdot}6$ were obtained for samples H and I, in agreement with their low viscosity.

Molecular weight of other polysaccharides

Xanthan samples X1, X3 and all samples from Satia (X5 to X11) were found identical in molecular weight, despite various changes in the process and differences in viscosity: $M_w = 2{\cdot}2 \times 10^6$, $I = 1{\cdot}4$. Sample X2 was lower, due to energetic grinding of the powder:

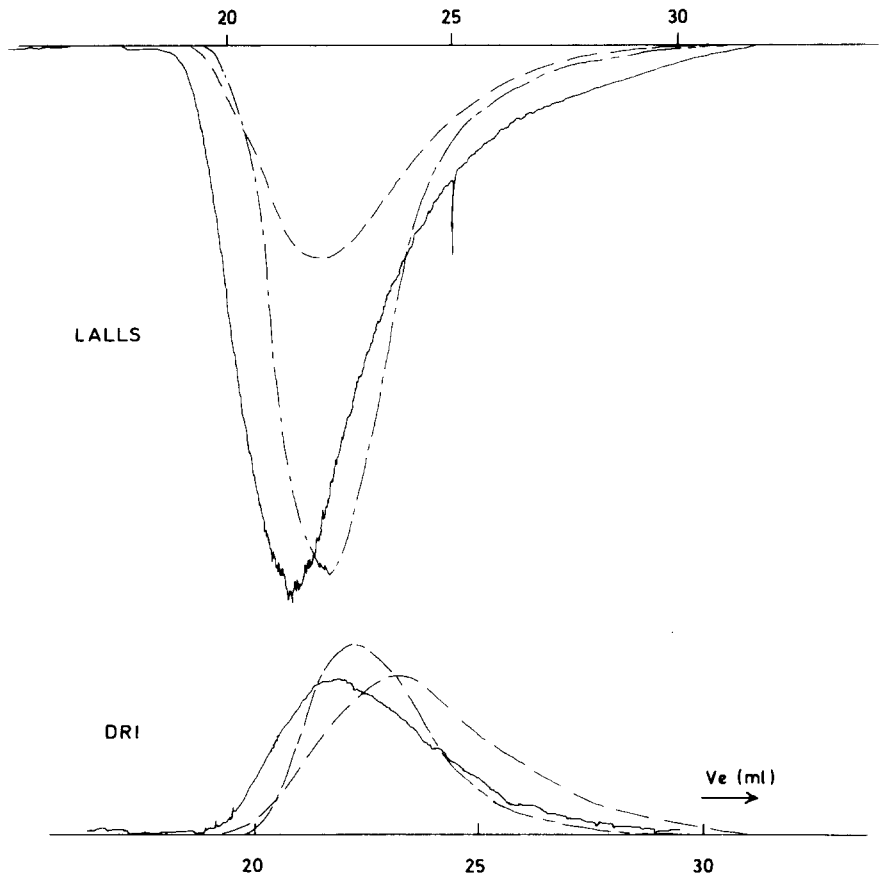

Fig. 6. Dual detector response for X4 (——), X11 (- - - -) and scleroglucan sample A (— — — —) (injected quantity 100 μg).

$M_w = 1{\cdot}6 \times 10^6$, $I = 1{\cdot}5$. X4 from Shell (UK) was considerably higher in molecular weight, which is clearly shown in Fig. 6 by comparing it to a typical xanthan sample X11 and to the scleroglucan sample A. A molecular weight $M_w = 7 \times 10^6$ was obtained, in good agreement with that of Milas (Milas *et al.*, 1985). Values for samples taken during a fermentation were as follows: $1{\cdot}22 \times 10^6$ for X12, $2{\cdot}57 \times 10^6$ for X13, $2{\cdot}75 \times 10^6$ for X14 and $2{\cdot}20 \pm 0{\cdot}01 \times 10^6$ for each of the last three samples.

Gellan samples from Kelco (USA) (G1 and G2) were found identical but of considerably lower molecular weight ($M_w = 150\,000$,

$I = 5$). The molecular weight of G3 was 560 000 ($I = 2$) and that of G4 was 300 000 ($I = 2$), which confirms that deacetylation of *Pseudomonas elodea* polysaccharide results in a splitting of molecular weight in half (Brownsey *et al.*, 1984). Finally, the molecular weight of new polysaccharide T was found to be close to xanthan: $M_w = 2 \times 10^6$, $I = 1{\cdot}2$.

DISCUSSION

This report has clearly shown the value of coupling SEC–LALLS in determining the molecular weight distribution of various microbial polysaccharides. Various difficulties usually encountered, the process of dissolution and filtration on one hand, and A_2 and dn/dc measurements on the other hand, have probably been overcome. These factors may easily explain the large discrepancies in results published by different laboratories. Our main results ($M_w = 2{\cdot}2 \times 10^6$ for xanthan and $M_w = 5{\cdot}7 \times 10^6$ for the triple helix of scleroglucan) are in agreement with the consensus that seems to emerge from the literature.

Scleroglucan samples, as well as xanthan, exhibited a constant molecular weight, despite large differences in their processing. With the exception of xanthan X4 from Shell, only a few samples had lower molecular weight than expected, but it is clear that a mechanical treatment was the reason for degradation. From the series J to L and X12 to X17, it was evident that the final molecular weight was quickly attained, at the middle of the period of fermentation or probably before. Such a constancy in the chain length could presumably be related to the mechanism of biosynthesis, but it is not easy to explain it further.

Polydispersity is often near unity. Recent results on succinoglucan (Gravanis, 1985) have confirmed this tendency. Significant deviation from unity, as observed for gellan gum, may be explained either by a limited stability of the powder or by a competitive depolymerization during biosynthesis.

There is no evident relationship between the molecular weight and the viscosity, η, of solutions. For xanthan samples, viscosity may vary from 1 to 4 (see Table 1) without change in molecular weight. This phenomenon has been clearly demonstrated (Milas & Rinaudo, 1984), as the same sample may adopt different conformations dependent on experimental conditions. Relative viscosities of sclero-

glucan solutions seem to be more stable, since low values of viscosity are mainly related to difficulties in solubilization. Therefore, molecular weight and viscosity measurements provide complementary information about microbial polysaccharides.

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