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The solution molecular weight and shape of the bacterial exopolysaccharides amylovoran and stewartan

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Abstract

Amylovoran, the acidic exopolysaccharide (EPS) of *Erwinia amylovora*, and stewartan, the capsular EPS of *E. stewartii*, were characterized by analytical ultracentrifugation and by size exclusion chromatography connected to dual detection of light scattering and mass. The average molecular weights of amylovoran and stewartan were determined as 1.0×10^6 and 1.7×10^6 Da, with polydispersity values (M_w/M_n) of 1.5 and 1.4, respectively. Based on the sugar composition and their molecular weight, both exopolysaccharides consist of approximately 1000 repeating units per molecule, this suggests a similar mechanism for chain length determination during biosynthesis of EPS in both organisms. © 1997 Elsevier Science B.V.

Keywords: Exopolysaccharide; Fire blight; Molecular weight determination

1. Introduction

The gram-negative bacterium *Erwinia amylovora* causes fire blight on apple and pear trees and other rosacious plants. In host plant tissue, the pathogen produces the exopolysaccharide (EPS) amylovoran, which is loosely associated with the bacterial cells. *Erwinia stewartii* causes Stewart's wilt on corn and produces the related EPS stewartan. The *cps*-mutants of *E. stewartii*, which are deficient in stewartan synthesis, are largely avirulent on corn seedlings [1], and the *ams*-mutants of *E. amylovora*, which lack synthesis of amylovoran, are non-pathogenic on pears or on apple seedlings [2]. The chromosomal region for biosynthesis of amylovoran has recently been characterized genetically and for its molecular organisation [3,4], and is related to the *cps*-region of *E. stewartii* [5,6]. In summary, these regions consist of 14 genes, which contain the genetic information for UDP-sugar transferases, functions for transport of the repeating units and

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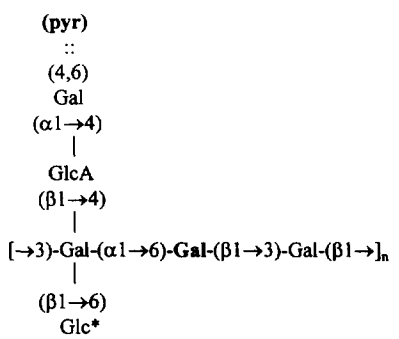
their polymerization and two genes for synthesis of UDP-galactose and UDP-glucose. It is possible to complement *E. stewartii* EPS-mutants with *E. amylovora* genes for amylovoran synthesis and vice versa [3,7]. The repeating units of amylovoran and stewartan consist of galactose, glucose and glucuronic acid residues [8,9] (Fig. 1). The backbone of amylovoran is a complex repeat consisting of three galactose residues; stewartan is similar except one galactose residue is substituted by a glucose residue. The side chains consist of glucuronic acid and galactose, which are terminated by pyruvate for amylovoran and glucose for stewartan. For a comprehensive physico-chemical analysis exceeding the unimer composition of these samples, the parameters of interest are: molecular weight distribution and molecular weight averages (principally M_w and M_n); degree of polymerization and the corresponding gross conformation in solution. These characteristics can be obtained from ultracentrifugation (sedimentation equilibrium and sedimentation velocity) on unfractionated material and further analysis of individual constituting components after separation by means of size exclusion chromatography (SEC) coupled on-line to a concentration detector and multi-angle light scattering (MALS).

(a)



(b)

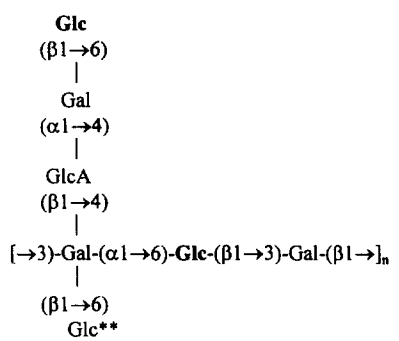


Fig. 1. (a) Repeating unit of amylovoran [8], * about 10% of repeating units with residue; (b) repeating unit of stewartan [9], ** about 90% of repeating units with residue. All sugars are in the D, *p*-configuration; differences between amylovoran and stewartan are printed in bold.

2. Materials and methods

E. amylovora strain Ea1/79 has been described by Falkenstein et al. [10]. DC283 is basically an *E. stewartii* wild type strain [5].

Amylovoran was routinely prepared from strain Ea1/79Sm (Ea1/79 with spontaneous resistance to streptomycin), which was grown in minimal medium MM2 [3]. After 2 days, the bacteria were removed by centrifugation, the supernatant concentrated in a Millipore apparatus, the concentrated solution spun in a preparative ultracentrifuge (Beckman rotor 60 Ti, 4 h at 40 000 rpm) and its supernatant extensively dialyzed against water. Finally, the solution was freeze dried and the EPS stored at room temperature. Stewartan was prepared from *E. stewartii* strain DC283, which was grown on CPG-agar, covered with cellophane disks. Cells and EPS were removed with 5 ml water per plate, centrifuged as above, dialyzed and freeze dried.

Samples were accurately weighed and left to dissolve overnight in a phosphate/chloride buffer made up using 4.60 $Na_2HPO_4 \times 12H_2O$, 1.56 g KH_2PO_4, 2.92 g NaCl in 1 l of distilled and deionised water.

Samples were dialysed in the above buffer prior to sedimentation equilibrium experiments.

2.1. Analytical ultracentrifugation

2.1.1. Determination of absolute weight average molecular weight (M_w) by sedimentation equilibrium

Sedimentation equilibrium experiments were performed at 20°C in a Beckman Model E analyt-

Table 1
Weight average molecular weights from SEC/MALS and sedimentation equilibrium and vbar and sedimentation coefficients for EPS samples

Sample	$\bar{v}$ (ml/g)	$s^0_{20,w}$ (S)	M_w SEC/MALS	M_w/M_n	$M_{w,app}$ sed. equil.
Amylovoran	0.639	9.2 ± 0.8	1.01×10^6	1.5	$(1.05 \pm 0.2) \times 10^{6a}$
Stewartan	0.664	14.7 ± 2.5	1.68×10^6	1.4	$(1.59 \pm 0.05) \times 10^{6a}$

[a] initial loading concentration: 0.8 mg/ml, rotor speed 3000 rpm.

ical ultracentrifuge fitted with a Rayleigh interference optical system and a rotor temperature indicator and control (RTIC) device. Twelve mm path length cells were assembled and filled with 100 μl sample and 120 μl solvent in the respective channels. Rotor speeds and concentrations used are indicated in Table 1. The light source was a 5 mW He–Ne laser. Interference fringes were recorded on photographic film and scanned on an Ultroscan Enhanced laser densitometer (LKB, Bromma, Sweden) Fringe shifts were obtained using the ANALYSER software [11] which produced an accurate record of concentration (in fringe units relative to the meniscus), $j(r)$, vs. radial displacement, r. ASCII data were then transferred to the routine MSTARI [12,13] for full molecular weight analysis: (i) concentrations (fringe units) $j(r)$ relative to the meniscus at $r = a$ were converted to absolute (fringe units) concentrations $J(r) = j(r) + J(a)$ using the procedure of Creeth and Harding [14] to find $J(a)$; (ii) the M^* function was used to obtain $M_{w,app}$, the weight average molecular weight over the solute distribution in the ultracentrifuge cell [14]; (iii) point weight average apparent molecular weights were obtained from local slopes of a plot of $\ln J(r)$ vs. r^2, using a sliding strip procedure [12,13].

The partial specific volume ($\bar{v}$) of the investigated samples was determined using the mechanical oscillator technique as described by Kratky et al. [15] using a precision digital densimeter (A. Paar, Graz, Austria).

2.2. Determination of sedimentation coefficient by sedimentation velocity

Experiments were carried out in a Beckman Model E analytical ultracentrifuge (Beckman, Palo Alto). Conditions such as cell path length, loading concentrations, rotor speeds and time intervals between photographs varied depending on the sample and are described in the text where appropriate. Photographs were developed and the sedimentation coefficients at experimental conditions ($s_{T,b}$) were evaluated using a graphics tablet and in-house software written by Dr A. Rowe and adapted by Dr H. Cölfen. $s_{T,b}$ was then converted to standard solvent conditions (viscosity and density of water at 20°C), $s_{20,w}$, according to

$$s_{20,w} = \frac{\eta_{T,b}}{\eta_{20,w}} \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{T,b}} \right) \times s_{T,b}$$

A plot of $s_{20,w}$ vs. concentration was constructed for each sample and the value for $s^0_{20,w}$ obtained from the intercept.

2.2.1. Determination of absolute molecular weight and molecular weight distribution by SEC/MALS

Size exclusion chromatography (SEC) separates molecules according to decreasing occupied volume, provided that there are no non-size exclusion mechanisms interfering with the separation. For a homologous series this results in a separation according to decreasing molecular weight. Dual detection with in-line mass and light scattering detectors allows determination of absolute molecular weight according to:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w(P\theta)} + 2A_2c \ldots$$

where K is the polymer constant for a particular scattering system, c is the sample (fraction) concentration, R_θ is the excess Rayleigh factor, M_w is the weight average molecular weight, $P(\theta)$ is the particle scattering function which contains information about particle dimensions (e.g. radius of

gyration, R_g), and A_2 is the second virial coefficient which is a measure of solvent–solute interactions and to a first approximation can be taken as zero due to the extremely low concentration of the individual sample fractions ($c \approx 1 \times 10^{-5}$).

The light scattering cell is illuminated by a 5 mW He–Ne laser (wavelength 633 nm) and the intensity of the scattered light is measured at 15 angles simultaneously. Weight average molecular weight for each fraction, $M_{w,i}$, is then obtained by extrapolation to zero angle using a plot of $R_{(\theta),i}/Kc_i$ vs. $\sin^2\theta/2$ (Debye plot [16]). Although the molecular weight at each slice is according to the above equation the weight average molecular weight, if the slices are assumed to be approximately monodisperse, then the number and z-averages over the whole distribution can be found from the usual equations [17]:

$$M_n = \frac{\sum c_i}{\sum (c_i/M_i)}$$

$$M_w = \frac{\sum c_i M_i}{\sum c_i}$$

$$M_z = \frac{\sum c_i M_i^2}{\sum c_i M_i}$$

2.3. Chromatographic conditions

The columns were eluted with the phosphate/chloride buffer described above. The SEC/MALS system consisted of a Waters 590 Solvent Delivery module (Waters, Millipore, Watford, UK), a Rheodyne injection valve (Model 7125) fitted with a 100 μl loop (Rheodyne, St Louis), a guard column and two analytical columns (Hema Bio linear and Hema Bio 40, PSS GmbH, Mainz, Germany) the latter consisted of a crosslinked hydroxyethyl methacrylate porous packing material, the first column having a linear separation range for dextrans of 7 million– < 2000, and the second column having a separation range below 40 000 (manufacturers values). Scattered light intensities were measured using a Dawn F multiangle light scattering photometer (Wyatt, Santa Barbara). Concentrations were measured using an Optilab 903 (Wyatt, Santa Barbara) interferometric refractometer. The eluent was pumped at a flow rate of 0.8 ml/min at ambient temperature and full loop injections of samples (1 mg/ml) were performed.

3. Results

The results obtained from our analyses are summarized in Table 1. Determination of the partial specific volume had to be performed prior to the sedimentation experiments as this value is needed for the calculation of molecular weights and sedimentation coefficients. The values obtained are in the range that would normally be expected for polysaccharides [18].

The sedimentation coefficients for the two samples were also found to be in the expected range. Both samples proved to be highly polydisperse which was indicated by their rapid dispersion into the buffer for the lower concentrations which gave rise to some uncertainties in the evaluation of the sedimentation coefficients at these concentrations. At higher concentrations the sedimentation profiles showed hypersharp peaks which are a sign for high (non-ideal) concentration dependence and polydispersity of the sedimentation coefficient [19]—this non-ideality is confirmed in the sedimentation coefficient vs. concentration plot for the amylovoran sample in Fig. 2. It appears that the stewartan preparation had the largest $s^0_{20,w}$ which would suggest either a higher molecular weight or a less extended conformation. However, the $s^0_{20,w}$ value for EPS of Ea1/79Sm was sufficiently low to be indicative of either a lower mass or a more extended conformation.

To remove the ambiguity of mass, molecular weights of the samples were determined using two absolute techniques—SEC/MALS and sedimentation equilibrium. The former provides a very rapid method for the determination of molecular weights and molecular weight distributions.

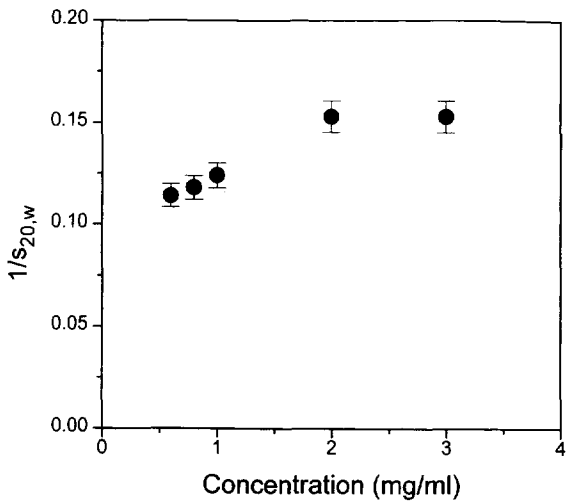


Fig. 2. Concentration dependence of sedimentation coefficient for amylovoran sample.

The values in Table 1 show that there is quite a large difference in molecular weights between the samples. The elution profiles of the main peaks (Fig. 3 for amylovoran) appear to be very similar for stewartan and amylovoran, both being quite narrow and having shoulders on the low molecular weight side. Both samples also contain some other low molecular weight component(s) at high elution volumes (15–20 ml) which are only detected by the concentration detector and were therefore not investigated any further. The molecular weight vs. elution volume plots (Fig. 4) are constructed from the molecular weights calculated over the whole (main) peak area and demonstrate the separation of the samples which in this case proved to be very good. It also shows the very pronounced shoulders for both samples indicating a very rapid decrease in molecular weight at the low molecular weight end of the peak. The molecular weight distributions in Fig. 5 very clearly demonstrate the difference in molecular weight and the molecular weight range for each sample. Polydispersity values (i.e. M_w/M_n, Table 1) of 1.5 and 1.4 must be considered relatively low and appear to contradict the interpretation of the sedimentation velocity results. However, the molecular weight distribution from SEC/MALS

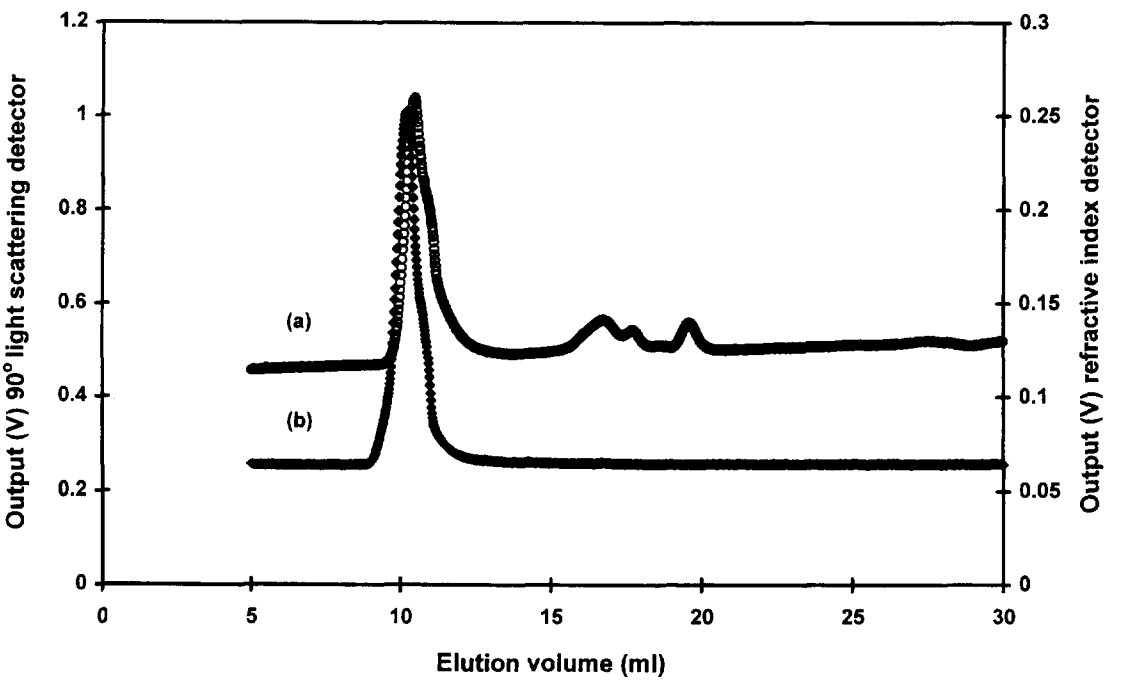


Fig. 3. Elution profile for amylovoran sample: (a) refractive index detector; (b) light scattering detector (90°). Experimental condidtions as described in text.

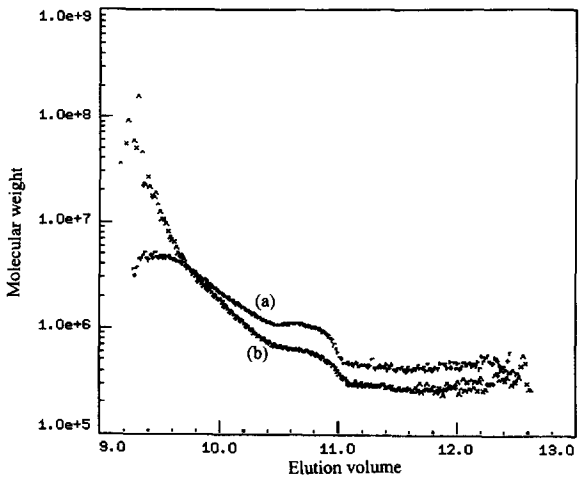


Fig. 4. Molecular weight vs. elution volume plot for (a) stewartan and (b) amylovoran.

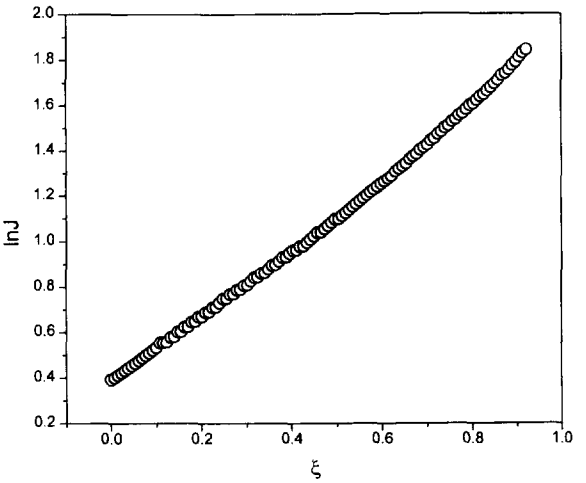


Fig. 6. Plot of ln J vs. ξ for amylovoran sample. Initial loading concentration 0.8 mg/ml, rotor speed 3000 rpm.

will always be 'compressed' due to the nature of the way in which the data was obtained. The fractions which are eluted from the column system are themselves not monodisperse, i.e. there is a molecular weight distribution in each slice and the detected molecular weight value at each slice is therefore strictly speaking the weight average molecular weight of that slice. The molecular weight distribution obtained will therefore appear to be narrower than the actual molecular weight distribution [20].

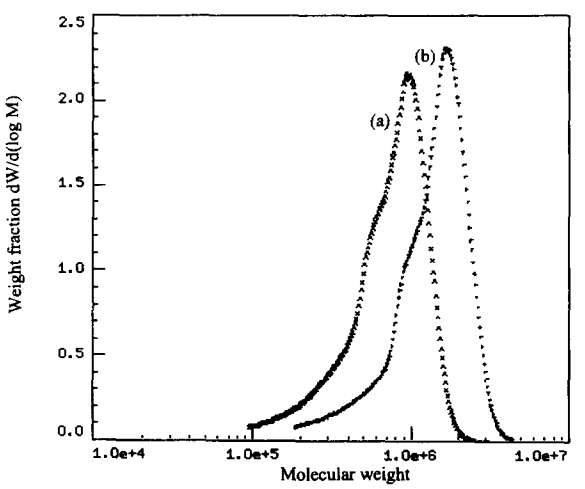


Fig. 5. Molecular weight distributions for (a) amylovoran and (b) stewartan.

The apparent weight average molecular weight ($M_{w,app}$) values obtained from sedimentation equilibrium under the experimental conditions were found to agree very well with the results from SEC/MALS (Table 1). Values for M_w (Table 1) were obtained from extrapolating the operational point average M^* to the cell base (Fig. 6) according to Creeth and Harding [14]. This method which considers the whole solute distribution in the centrifuge cell is generally better suited for evaluating M_w of polydisperse materials compared with the conventional extrapolation of the concentration to the cell base and meniscus and does not require an estimate of the initial loading concentration. The assumption was made that at the very low concentrations used (0.8 mg/ml) non-ideality is small and $M_w \approx M_{w,app}$.

Plots of ln J vs. ξ were found to be linear (Fig. 6 for amylovoran) and the corresponding plots of point $M_{w,app}(r)$ vs. J (Fig. 7 for stewartan) were approximately flat, suggesting that the effect of what non-ideality there is, is matched by the polydispersity of the samples [21,22].

4. Discussion

The molecular weights and degrees of polymerization (D_p) found by the different techniques agreed very well giving values of $D_p \sim 1000$ and ~ 1200 for amylovoran and stewartan respectively. Although molecular weights have been reported previously a more extensive study of the hydrodynamic behaviour of amylovoran and stewartan has not been carried out, to our knowledge. Molecular weights had previously been estimated at 50–150 mDa (for amylovoran) and ~ 45 mDa (for stewartan) [23]. These values are much higher than those found by us but this may be a reflection of the technique used for the determination of molecular weight. Calibrated techniques in particular are notoriously unreliable for the molecular weight determination of polysaccharides.

Amylovoran and stewartan are both branched, acidic polysaccharides and this would suggest that these molecules will adopt a more compact conformation in solution than neutral, linear molecules. In addition, the samples were dissolved in a buffer of ionic strength of 0.1 to shield the charges thus further encouraging a compact conformation. This was confirmed by the sedimentation coefficients found (i.e. 9.2 and 14.7 for amylovoran and stewartan respecitvely) which are considerably larger than for extended polysaccharides of similar molecular weight like for example guar gum (a neutral galactomannan) which has been reported to have a $s^0_{20,w}$ value of ~ 5 S [24].

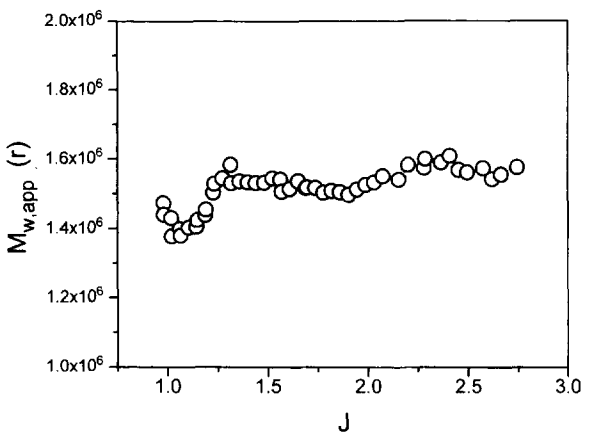


Fig. 7. Plot of $M_{w,app}(r)$ vs. J for stewartan sample. Experimental conditions as in Fig. 6.

Erwinia amylovora and *Erwinia stewartii* are strictly dependent on EPS synthesis in order to cause disease symptoms [3–5]. A strong effect on the host plants is plugging of xylem vessels and disruption of water flow [25]. Suggestions have also been made, that the EPSs may help the invading bacteria to avoid the plant's defense mechanisms by preventing recognition between plant cell walls and the parasite [26,27]. The hydrodynamic behaviour described above would fit in very well with the former scenario, EPS molecules being sufficiently compact to move through the vessels to the site of attack but also sufficiently large and hydrophilic to swell to a large volume and disrupt the water flow.

5. Conclusions

The results obtained from sedimentation equilibrium, sedimentation velocity and SEC/MALS experiments for amylovoran and stewartan are in good agreement. The molecular weight determinations were done with single preparations of amylovoran and stewartan. EPS from preparation of two *E. Amylovoran* strains or two *E. Stewartii* strains can display molecular weights, which differ by less than 10% (A. Huber, and K. Geider, unpublished results). On the other hand, purification of stewartan by column chromatography resulted in a significantly lower molecular weight EPS than found from preparations without this step. We assume preferential elution of short molecules from an ion exchange column. An influence of the growth temperature for the bacterial cultures or composition of growth media is not known. On the other hand, molecular weight values were not found to be as large as reported by other authors, however, the results indicated that the molecules appear to adopt a relatively compact conformation under the conditions employed.

Acknowledgements

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