Carbohydrate Polymers **23** (1994) 55–64

Solution properties of levan polysaccharide from *Pseudomonas syringae* pv. *phaseolicola*, and its possible primary role as a blocker of recognition during pathogenesis

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(Received 28 April 1993; revised version received 16 August 1993; accepted 19 August 1993)

Bacterial levan, a highly branched, high molecular weight polymer of fructose, was purified from culture supernatants of *Pseudomonas syringae* pv. *phaseolicola* grown in a liquid high-sucrose medium, and the predominance of β-(2 → 6) linkages was confirmed by ^{13}C NMR. The solution properties of this material resembled those of disordered linear polysaccharides in the response to low-amplitude oscillatory shear (frequency dependence of G' and G''); the absence of any detectable conformational change with temperature (as monitored by optical rotation); close superposition of steady-shear viscosity (η) and complex dynamic viscosity (η^*) at equivalent values of shear-rate ($\dot{\gamma}$/s^{-1}) and frequency (ω/rad s^{-1}); a similar form of shear-thinning (giving linear plots of η *versus* $\eta\dot{\gamma}^{0.76}$); and the onset of semi-dilute behaviour at a closely comparable degree of space-occupancy ($c[\eta] \approx 3{\cdot}6$). The intrinsic viscosity, however, was unusually low ($[\eta] \approx 0{\cdot}17$ dl g^{-1}) and the concentration dependence of 'zero-shear' viscosity in the semi-dilute regime unusually high ($\eta_0 \sim c^{9.3}$), as anticipated from the densely packed, branched molecular structure.

Solutions of levan and pectin, matched to approximately the same initial viscosity, showed a substantial reduction in viscosity when mixed. Similar behaviour was observed for mixed solutions of levan with locust bean gum (LBG), chosen for its structural similarity to cellulose and hemicelluloses of the plant cell wall. Viscosity reduction was eliminated at low concentrations (indicating that it does not arise from heterologous association), but became very pronounced at high concentrations, and was then accompanied by resolution into levan-rich and LBG-rich solution phases. This evidence of strong thermodynamic incompatibility and exclusion behaviour with (1 → 4)-linked plant polysaccharides suggests that the primary role of levan during pathogenesis may be as a barrier to intimate morphological contact (recognition) between plant cell walls and those of the parasite, thus inhibiting initiation of a hypersensitive response by the host.

INTRODUCTION

Levan is produced from sucrose by many plant-associated and plant-pathogenic bacteria (Starr *et al.*, 1981). In particular, bacteria that propagate within the intercellular space of the mesophyll tissue and cause 'leaf spotting', synthesise copious amounts of levan when cultured *in vitro* (Gross & Rudolph, 1987*a*, *b*, *c*), behaviour which has been long known (Cooper & Preston, 1935) and used as a taxonomic criterion in phyto-

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Carbohydrate Polymers 0144-8617/94/$07.00

bacteriology (Schaad, 1988). Appreciable amounts of levan can also be extracted from severely diseased leaf tissues, and are present in the exudates that frequently emerge from the centres of infection sites (Gross & Rudolph, 1987*d*).

Chemically, levan consists of β-D-fructofuranosyl residues linked predominantly through (2 → 6), but with extensive branching through (2 → 1) linkages (Stivala & Khorramian, 1982; Allen & Bowen, 1990). The molecular weights of bacterial levans are typically in the range (2–100) × 10^6 (Keith *et al.*, 1991) with the final molecular size being greatly influenced by the conditions (ionic strength, temperature, co-solutes, etc.) under which the synthesis takes place (Tanaka *et al.*, 1978, 1979). The molecular shape, as visualised by electron microscopy (Newbrun *et al.*, 1971), is spheroidal, indicating that the constituent chains are extended radially at the same synthetic rate.

As far as is known, bacterial levan is synthesised by a single exoenzyme, levansucrase (E.C. 2.4.1.10), whose preferred substrate is sucrose. Sucrose is the predominant form in which sugar is transported in assimilating plants, and is therefore present in appreciable concentrations in the intercellular space of most plant tissues. Levan synthesis occurs by a transfructosylation process which yields monomeric glucose in addition to the high molecular weight fructan. The levansucrase enzyme is rather small (45 kD), and can act autonomously, without requiring any co-factors or external energy other than that conserved in the acetal linkage of sucrose itself. It is also highly tolerant to physical and chemical stress (U. Hettwer, unpublished), and therefore very well adapted to surviving the hostile environment presented by the interior of a living host.

Although it can well be imagined that such an exoenzyme diffusing in advance of the bacterial attack might create favourable conditions for establishment of the parasite, its detailed role during pathogenesis has not yet been elucidated, nor that of its product, levan. In the present work, we have attempted to gain some further insight into the possible biological function of bacterial levan by detailed characterisation of its rheological properties *in vitro*, and by investigating its interaction with representative plant polysaccharides.

MATERIALS AND METHODS

Bacterial levan

Levan was produced by cultivating *Pseudomonas syringae* pv. *phaseolicola* (strain Ex-4) in a high-sucrose (5% (w/v)) liquid medium, and purified as described by Gross and Rudolph (1987*c*). Briefly, the cultures were harvested after two days, by centrifugation. The macromolecular fraction of the supernatant was obtained by high-capacity cross-flow ultrafiltration (PTHK membrane; 100 kD nominal molecular weight limit; Pellikon equipment, Millipore). Low molecular weight materials were removed by reverse osmosis; protein, acidic polysaccharide (alginate) and LPS (lipopolysaccharide) were absorbed by passage through a DEAE–fractogel column. The yield of purified levan, after final molecular washing and lyophilisation, was about 36 g from 3 litres of culture supernatant, in agreement with previous studies (Gross & Rudolph, 1987*c*).

The purity of the product was assessed using the procedures detailed by Gross and Rudolph (1987*a*). Protein could not be detected by colourimetric assays (Lowry *et al.*, 1951; Bradford, 1976), but when very concentrated solutions were run in an SDS-PAGE system and developed with a highly sensitive activity-staining procedure (U. Hettwer, unpublished) trace amounts of residual levansucrase were observed. Analysis of TFA-hydrolysed preparations by thin-layer chromatography, however, showed no evidence of contamination with alginate or LPS, even under overloading conditions. The predominance of β-(2 → 6) linkages, as expected for a bacterial levan, was confirmed by ^{13}C-NMR (70°C in D_2O, recorded at 125 MHz on a Varian VXR-500 spectrometer), yielding a spectrum closely similar to that observed previously (Gross *et al.*, 1992) for levan from *Erwinia amylovora*.

Solutions for rheological and chiroptical measurements were prepared in deionised water by mechanical stirring (typically for 10 min at 70°C).

Interactions with plant polysaccharides

Pectin and locust bean gum (LBG) were chosen as representative of the predominant linkage-patterns in plant polysaccharides. Interactions with bacterial levan in solution were monitored by the procedure suggested by Morris (1984). This involves bringing solutions of the two polymers under investigation to approximately the same viscosity and mixing them in various ratios, with any departures from the initial common viscosity of the starting solutions giving evidence of polymer–polymer interaction.

Stock solutions of levan, pectin (high-methoxy rapid-set; Bulmers) and LBG (Rex 5924; Marine Colloids Division of FMC) were prepared in a 50 mM sodium phosphate buffer (pH 7·0) containing 500 ppm sodium azide as preservative, and dialysed overnight against the same buffer. Individual solutions were then matched to the same desired viscosity by appropriate dilution with the dialysate, clarified by passing through a 3 μm Millipore SSWP-filter, and mixed in ratios (v/v) of 1:9, 2:8, 4:6, 6:4, 8:2 and 9:1. Any air bubbles introduced during mixing were removed by brief centrifugation, and solution viscosity was measured as detailed below.

Physical techniques

Steady-shear viscosity measurements were made on a Contraves Low-Shear 30 rotational viscometer, using cup-and-bob geometry with inner and outer radii of 5·5 and 6·0 mm, respectively. Temperature was controlled to within ±0·5°C by a circulating water bath and measured by a thermocouple in direct contact with the sample.

Small-deformation oscillatory measurements were performed on a Sangamo Viscoelastic Analyser, using cone-and-plate geometry of 2° cone angle and 5 cm diameter. The same instrument was also used to measure viscosities above the range of the Contraves viscometer. Temperature was controlled and measured as described above.

Optical rotation was measured on a Perkin-Elmer 241 polarimeter, using jacketed cells of 1 cm or 10 cm path length, as appropriate. Temperature was again controlled to within ±0·5°C, and measured using a thermocouple in the neck of the cell, in contact with the sample but out of the light path.

INTRINSIC VISCOSITY OF LEVAN

The viscosity generated by polymers in dilute solution provides a convenient route to characterising their hydrodynamic volume. The parameter most readily obtained from experimental measurements is the relative viscosity (η_{rel}), the ratio of solution viscosity (η) to solvent viscosity (η_s). A more meaningful index, however, is the specific viscosity (η_{sp}), which defines the fractional increase in viscosity due to the presence of the polymer ($\eta_{sp} = (\eta - \eta_s)/\eta_s = \eta_{rel} - 1$). The ratio of specific viscosity to concentration (i.e. the 'viscosity number', η_{sp}/c) then gives a measure of the effectiveness of the polymer molecules in perturbing the flow of the solvent, and hence of their size.

The viscosity number, however, is not constant, but increases with concentration due to interactions between neighbouring chains. This complication can be overcome by extrapolating experimental values to zero concentration, to yield the limiting viscosity number, or 'intrinsic viscosity', $[\eta]$, which gives a direct index of the volume occupied by the individual polymer molecules in isolation. The extrapolation is linear if confined to relative viscosities below about 2·0, with experimental precision normally imposing a lower limit of $\eta_{rel} > 1{\cdot}2$.

Relative viscosities within this range were obtained for levan at concentrations between about 1 and 3% (w/v). As shown in Fig. 1, the 'Huggins plot' of η_{sp}/c *versus* c is acceptably linear, yielding an intrinsic viscosity of $[\eta] \approx 0{\cdot}17\,\mathrm{dl\,g^{-1}}$ (at 20°C). As would be anticipated from the very compact, highly branched molecular structure of levan, this value is exceptionally low. By comparison, typical intrinsic viscosities for

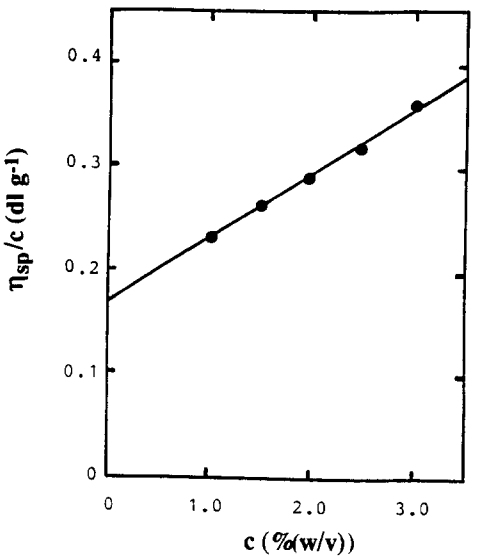

Fig. 1. Huggins plot for bacterial levan (20°C), yielding $[\eta] \approx 0{\cdot}17\,\mathrm{dl\,g^{-1}}$.

linear polysaccharides (Morris & Ross-Murphy, 1981) range from $[\eta] \approx 1\,\mathrm{dl\,g^{-1}}$ for very flexible chains (e.g. dextran) or very contorted, compact coils (e.g. amylose) to $[\eta] \approx 20\,\mathrm{dl\,g^{-1}}$ for extended chains (e.g. alginate, galactomannans).

SHEAR THINNING

The solution viscosity of levan remained Newtonian (i.e. independent of shear rate) up to a polymer concentration of about 20%. Significant shear-thinning (i.e. reduction in viscosity with increasing shear rate) was observed (Fig. 2) at higher concentrations, but a 'plateau region' of Newtonian flow at low shear rates remained readily accessible up to the highest concentration at which solutions could be prepared (26·5%).

Qualitatively similar behaviour is well documented and well understood for linear polymers as disordered coils in solution. At low concentration, in the 'dilute-regime' where, as discussed above, individual polymer molecules contribute to viscosity by disrupting the flow of the solvent, any shear-thinning effects are slight (seldom more than 30%) and can be attributed to some limited alignment and stretching of the polymer chains in the direction of flow. At higher concentration, however, the individual coils are forced to interpenetrate one another, and form an entangled network (Graessley, 1974). Shear thinning in this 'semi-dilute' regime occurs in an entirely different way. To allow the solution to flow, overlapping chains must be pulled apart. If the rate of movement is sufficiently low, this process is balanced by interpenetration of different chain-partners, giving rise to the characteristic. Newtonian plateau at low shear rates. At higher shear rates,

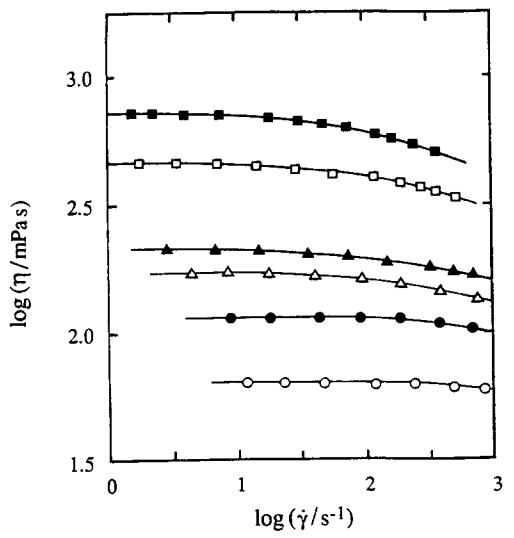

Fig. 2. Shear-rate ($\dot{\gamma}$) dependence of viscosity (η) at 20°C for levan at concentrations (% (w/w)) of: 20·4 (○), 21·5 (●), 22·5 (△), 23·5 (▲), 25·0 (□) and 26·5 (■).

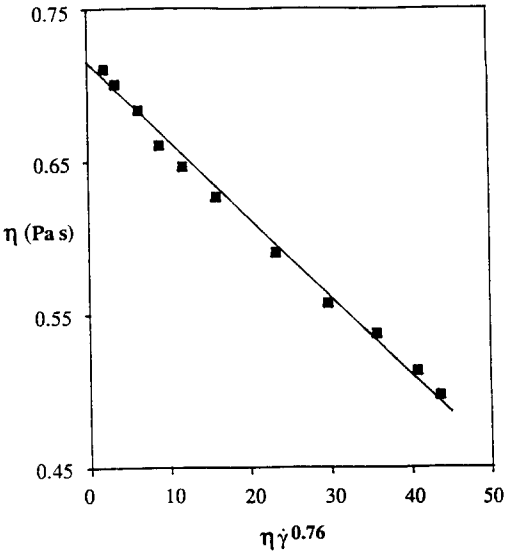

Fig. 3. Linearised shear-thinning plot (Morris, 1990) for levan (26·5% (w/w)) at 20°C.

where re-entanglement can no longer keep pace with forced disentanglement, the network is depleted and viscosity falls, often by two orders of magnitude, or more, over the shear-rate range accessible on typical commercial viscometers.

The reduction in viscosity (η) with increasing shear rate ($\dot{\gamma}$) in such systems can be fitted with good precision (Morris, 1990) by the simple relationship

$$\eta = \eta_0/[1 + (\dot{\gamma}/\dot{\gamma}_{1/2})^p] \quad (1)$$

where η_0 is the constant, maximum viscosity in the Newtonian plateau region and $\dot{\gamma}_{1/2}$ is the shear rate required to reduce η to $\eta_0/2$. Equation (1) can be recast in linear form as:

$$\eta = \eta_0 - (1/\dot{\gamma}_{1/2})^p \eta \dot{\gamma}^p \quad (2)$$

The exponent, p, corresponds to the maximum (negative) gradient of log η *versus* log $\dot{\gamma}$ at high shear rates, and is found experimentally to have a constant value of 0·76 for disordered linear polysaccharides of high polydispersity (as in all normal preparations). Thus plotting η *versus* $\eta\dot{\gamma}^{0.76}$ for such systems gives straight lines of gradient - $(1/\dot{\gamma}_{1/2})^{0.76}$ and intercept η_0. When the same treatment is applied to systems such as xanthan 'weak gels' (Ross-Murphy *et al.*, 1983), where the polymer chains interact enthalpically through non-covalent associations, the resulting plots show pronounced curvature (Morris, 1990).

At all concentrations where shear thinning was observed (Fig. 2), the results for levan gave reasonable linearity ($r^2 > 0.98$) over the full range of accessible shear rates, as in the illustrative plot shown in Fig. 3, and the intercepts on the vertical axis were in good agreement with the values of η_0 observed directly at low shear rate.

Although the exponent of 0·76 was derived empirically (Morris *et al.*, 1981), it does have an underlying theoretical basis (Ferry, 1980) in the rate at which linear chains can recover ('relax') from a forced change in shape. Highly branched polymers such as levan would be expected to give a somewhat different exponent because of differences in the underlying intramolecular relaxation spectra, but the extent of shear-thinning (Fig. 2) is insufficient to allow the precise value of p for levan to be determined reliably.

The central conclusion from the analysis shown in Fig. 3, however, is that the response of levan to changes in shear-rate is close to that observed for disordered linear chains interacting solely by physical entanglement, with no evidence of the grossly different shear thinning seen in systems where entanglement is augmented (or replaced) by non-covalent association.

CONCENTRATION DEPENDENCE OF VISCOSITY

The concentration dependence of the 'zero-shear' Newtonian viscosity (η_0) for levan is shown in Fig. 4 as a double-logarithmic plot of η_{sp} *versus* c. The results fall into three linear regions, with changes of gradient at polymer concentrations of about 4 and 20%. As described above, the shear-thinning behaviour characteristic of semi-dilute solutions is confined to the high-concentration region ($c > 20\%$). The low-concentration region ($c < 4\%$) obviously corresponds to the dilute solution regime, and has a gradient the same as that observed (Morris *et al.*, 1981) for dilute solutions of unbranched polymer coils (~1·4). The first transition, marking the end of the dilute regime, is attributed to initial contact

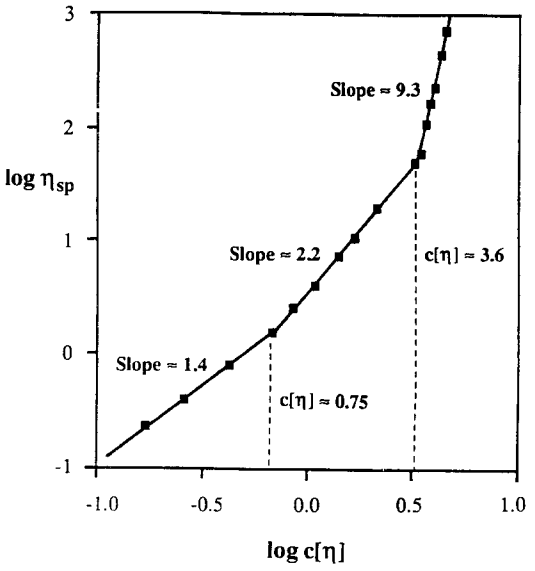

Fig. 4. Concentration-dependence of 'zero-shear' specific viscosity for levan at 20°C.

between the individual molecules when, in aggregate, their swept-out volume becomes equal to the total volume of the solution; the second corresponds to the onset of long-range entanglement.

The concentrations at the beginning and end of the intermediate zone are conventionally designated as, respectively, c^* and c^{**} (see, for example, Milas *et al.* 1990). The precise values of both are somewhat dependent upon the method used for their determination, since different techniques may differ widely in their sensitivity to changes in molecular mobility. However, initial contact normally occurs when the (dimensionless) product of concentration (proportional to the number of polymer molecules present) and intrinsic viscosity (proportional to the volume that each occupies) reaches a value of $c[\eta] \approx 1$ (i.e. $c^* \approx 1/[\eta]$); values reported for $c^{**}[\eta]$ vary with system and technique from around 2 to over 10.

In comparison with other techniques such as dynamic light scattering (e.g. Southwick *et al.*, 1979) and fluorescence-bleaching measurements of self-diffusion (Tinland *et al.*, 1990) viscosity measurements are normally rather insensitive to the progressive increase in overlap of polymer coils between c^* and c^{**}. In particular, plots of log η_{sp} *versus* log c for disordered linear polysaccharides approximate closely to two straight lines with a sharp change in gradient from ~1·4 to ~3·3 at $c[\eta] \approx 4$ and $\eta_{sp} \approx 10$, although some slight curvature may be observed around the 'break-point'. It should be noted that in the paper first documenting this behaviour (Morris *et al.*, 1981), and in several subsequent publications (e.g. Pereira *et al.*, 1982; Rees *et al.*, 1982; Morris, 1984), the concentration at the intersection of the two linear regions was denoted as c^*. In terms of the more conventional usage outlined above, however, the concentration at this point will lie between c^* and c^{**}, probably closer to the latter, and will be referred to here as c_{crit} ($\approx 4/[\eta]$).

A somewhat better-defined intermediate region has been reported for xanthan (Launay *et al.*, 1984; Milas *et al.*, 1990), which exists in solution in a rigid, ordered chain conformation (Norton *et al.*, 1984), but the experimental points still lie very close to the two straight lines used by Morris *et al.* (1981) to fit their results for disordered chains; the difference is largely one of interpretation rather than reflecting any significant difference in solution properties.

The results reported here for levan, however, show very major departures from those obtained for linear polysaccharides. Since the intrinsic viscosity was measured as $[\eta] = 0{\cdot}17\,\mathrm{dl\,g^{-1}}$, the changes in gradient shown in Fig. 4 occur at $c[\eta] \approx 0{\cdot}7$ and $c[\eta] \approx 3{\cdot}6$, which, as discussed above, are reasonable values for the c^* and c^{**} transitions, respectively. The specific viscosities at $c < c^*$ are very close to those of linear chains at the same degree of space-occupancy (i.e. at equivalent values of $c[\eta]$), with $\eta_{sp} \approx 1{\cdot}5$ at c^*. After c^*, where the gradient of log η_{sp} *versus* log c increases from ~1·4 to ~2·2, the viscosities rise above those of the equivalent unbranched-polysaccharide systems, reaching $\eta_{sp} \approx 50$ at c^{**}.

The most striking divergence, however, occurs at $c > c^{**}$, where the gradient increases to about 9·3 (so that doubling the concentration would increase η_0 by a factor of about 600, in comparison to the 10-fold increase observed for linear chains).

TEMPERATURE DEPENDENCE OF OPTICAL ROTATION

One possible interpretation of the extreme concentration dependence of levan viscosity would be the formation of cooperative intermolecular junctions, as in gelling systems, rather than (or in addition to) physical entanglement. Enthalpically stabilised associations of this type are often thermally labile, with their dissociation and re-formation being accompanied by abrupt changes in optical rotation on heating and cooling (Rees *et al.*, 1982).

Experimental results for levan, however, showed no evidence of any such effects. The temperature dependence of optical rotation (see, e.g., Fig. 5) remained essentially linear throughout the experimentally accessible range of levan concentration.

VISCOELASTICITY

A more direct way of probing the nature (and timescale) of long-range intermolecular association in polymer

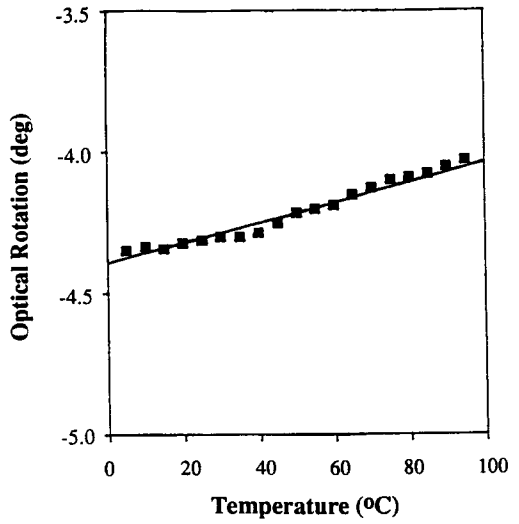

Fig. 5. Temperature-dependence of optical rotation (365 nm; 1 cm path length) for a semi-dilute solution of levan (25% (w/w)).

systems is by mechanical spectroscopy (Ross-Murphy, 1984). The sample under investigation is subjected to a low-amplitude oscillatory deformation and the resistance resolved into an in-phase component, characterising elastic (solid-like) response and quantified as the 'storage modulus' (G'), and an out-of-phase component which quantifies viscous flow as the 'loss modulus' (G''). The ratio of the unresolved 'complex modulus' (G^*) to the frequency of oscillation (ω) gives the 'complex dynamic viscosity' (η^*).

$$\eta^* = G^*/\omega = (G'^2 + G''^2)^{1/2}/\omega \qquad (3)$$

Figure 6 shows a typical mechanical spectrum (frequency dependence of G', G'' and η^*) for levan

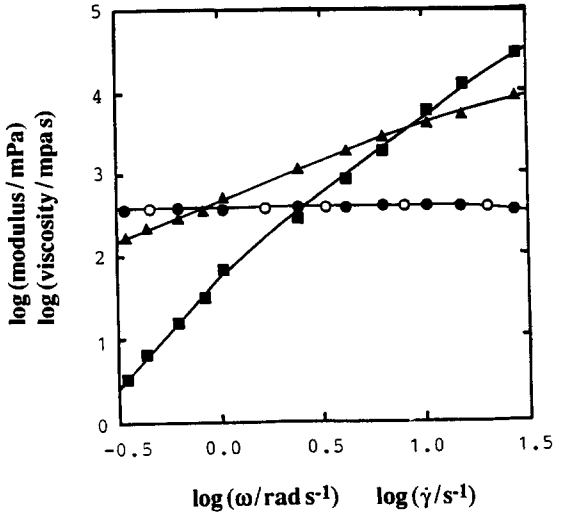

Fig. 6. Frequency (ω) dependence of G' (■), G'' (▲) and η^* (●) and shear-rate ($\dot{\gamma}$) dependence of η (○) for a typical semi-dilute solution of levan (25·0% (w/w)) at 20°C.

under semi-dilute conditions (at 25%). The spectrum is closely similar to those obtained for entangled linear polysaccharides (Morris, 1984). At low frequencies, where substantial disentanglement can occur within the period of oscillation, viscous response (G'') predominates. At higher frequencies, where there is less time for network rearrangement, the response becomes more like that of a gel, with elastic character (G') predominating.

A further point of similarity is that levan solutions, like those of disordered linear polymers, obey the 'Cox–Merz' rule (Cox & Merz, 1958); as illustrated in Fig. 6, steady-shear viscosity (η) and dynamic viscosity (η^*) are closely superimposable at equivalent numerical values of shear-rate ($\dot{\gamma}/s^{-1}$) and frequency (ω/rad s^{-1}). This behaviour is general for systems where no energy is required to disrupt the associations leading to long-range structure.

The response of 'weak gel' networks stabilised by non-covalent bonding between neighbouring chains (e.g. semi-dilute solutions of xanthan) is quite different (Ross-Murphy *et al.*, 1983). Such networks remain intact under the small-deformation oscillatory conditions used in mechanical spectroscopy, but are broken down by unidirectional shear, giving $\eta(\dot{\gamma}) < \eta^*(\omega)$. Thus the viscoelastic properties of levan reinforce the indication from its shear-thinning behaviour (Fig. 3) that the intermolecular interactions in semi-dilute solution are topological rather than enthalpic.

INTERACTION WITH PLANT POLYSACCHARIDES

The results presented so far have demonstrated that bacterial levan displays some unusual rheological features which may be of relevance to understanding its biological role in pathogenesis. The studies reported here address its possible interaction with surface polysaccharides of the plant cell wall.

One of the major cell-wall constituents (Albersheim, 1974) is pectin, which is readily available in purified, soluble form. The pectin backbone consists predominantly of *a*-D-galacturonate residues (a proportion of which are methyl-esterified) linked through axial bonds at positions 1 and 4 of the pyranose ring. The other major cell-wall polysaccharides are cellulose and the hemicelluloses, where the linkages are again predominantly (1 → 4), but diequatorial rather than diaxial, giving a flat, extended chain profile in comparison with the buckled chain geometry of pectin. Because of the obvious problems of solubility and/or availability, we have not studied these materials directly, but have used the soluble storage-polysaccharide locust bean gum (LBG), which shares the same (1 → 4)-diequatorial linkage geometry and would therefore be expected (Rees *et al.*, 1982) to behave similarly, both in solution properties and in binding interactions with other chains.

Figure 7 shows the results obtained when a solution of levan (at a concentration just above c^{**}) was mixed in various ratios with a pectin solution of approximately the same viscosity. In the absence of any interaction between the two polymers, the viscosity would be expected to remain close to the common value for the individual starting solutions. Direct association to form crosslinked 'microgel' particles can give very large increases in viscosity, as seen, for example, in mixed solutions of xanthan with plant galactomannans (Morris *et al.*, 1980). In the levan–pectin system, by contrast, a substantial reduction was observed (Fig. 7), with viscosity at some mixing ratios dropping to less than half that of the constituent solutions. The measured viscosities showed little change over a wide range of composition, which again contrasts with the sharp maxima commonly obtained in systems where viscosity is enhanced by specific heterotypic association.

Similar behaviour was observed for mixed solutions of levan with LBG. As illustrated in Fig. 8, viscosity reduction became progressively less apparent as polymer concentration was decreased, with sufficiently dilute solutions ($\eta_{rel} \approx 2$) showing the invarient viscosity anticipated in the absence of any interaction between the two polymers. At the higher concentrations where viscosity suppression was observed, the mixed solutions had a 'streaky' appearance, indicative of macroscopic heterogeneity. The striations persisted on prolonged stirring, and on centrifugation the solutions separated into two liquid layers, the lower one consisting predominantly of the denser polymer (levan).

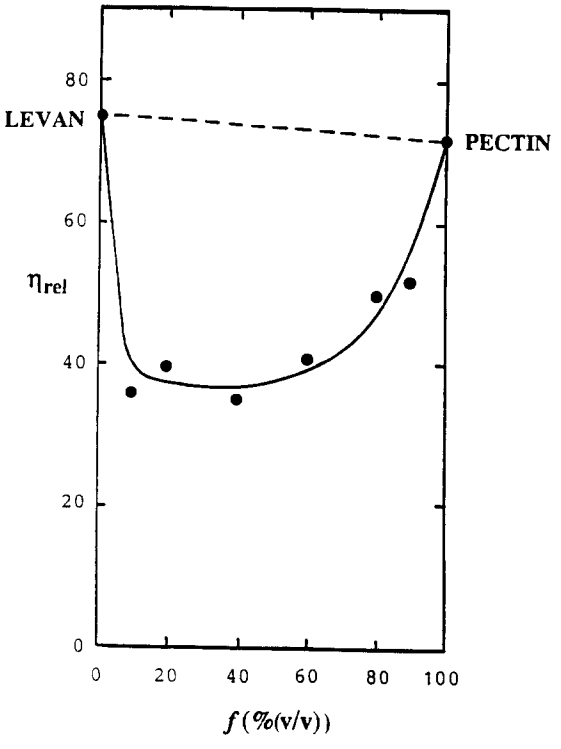

Fig. 7. Suppression of viscosity (20°C; 10 s^{-1}) in mixed solutions of levan with pectin; f denotes the proportion of pectin solution in each mixed system, with $f = 0$ and $f = 100\%$ (v/v) corresponding to the starting solutions of levan and pectin, respectively.

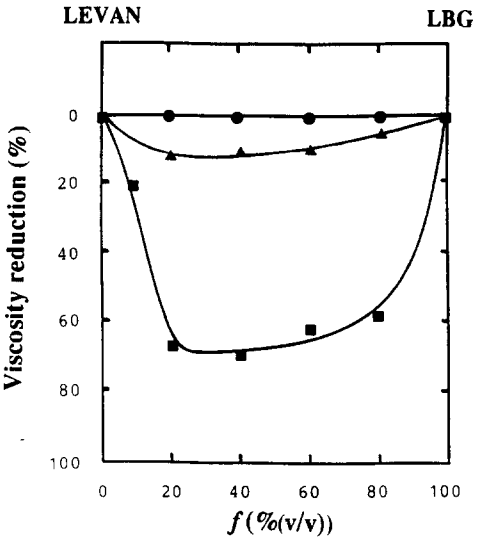

Fig. 8. Suppression of viscosity in mixed solutions of levan with LBG. Results are shown for combinations of stock solutions with relative viscosities of 2 (●), 15 (▲) and 108 (■). In each case f denotes the proportion of LBG solution in the mixed systems.

Phase-separation phenomena of this type are common in mixed polymer systems (Tolstoguzov, 1986) and their origin is well understood. There is normally an enthalpic advantage in polymer segments being surrounded by others of the same type, rather than by chain segments of a different type, so that in mixed solutions each polymer tends to resist interpenetration by molecules of the other. At low concentrations, the effect of this mutual exclusion is to drive the chain into a more compact form. In particular, if one component can undergo a conformational transition from an expanded coil to a compact ordered structure, the presence of the other will promote the disorder–order transition. At higher concentrations, the two polymers may separate into discrete phases, as observed here for mixed solutions of levan and LGB.

Thermodynamic incompatability between dissimilar materials is not, of course, confined to polymers, but for small molecules it is normally swamped by the entropic advantage of different species remaining mixed together in a single phase. Entropy of mixing is much less significant in polymer systems, since the number of solute molecules is far smaller at equivalent weight-concentrations, but it is reflected in the presence of a small, equilibrium concentration of each polymer in the phase where the other predominates.

Immediately after mixing, one phase will normally be dispersed as small liquid droplets, with the other forming a continuous surrounding matrix. If the two phases

differ in density, the droplets of the dispersed phase will gradually rise or sink through the continuous phase, eventually coalescing into a separate liquid layer. This process can, of course, be accelerated by centrifugation, as in the present work.

The most likely interpretation of the results shown in Figs 7 and 8 is that comparatively small concentrations of pectin or LBG are sufficient to drive levan into a separate dispersed phase, leading to the sharp decrease in viscosity observed at low mixing ratios of plant polysaccharide:levan, with the recovery of viscosity at higher mixing ratios reflecting the increasing concentration of plant polysaccharide in the continuous phase. We are currently investigating these systems in greater detail, to obtain phase diagrams that can be related quantitatively to solution rheology. For the moment, however, it is clear that the bacterial levan produced by plant pathogens shows striking exclusion effects with polysaccharides typical of those at the surface of plant cells.

DISCUSSION

Solution rheology of levan

Despite its highly branched molecular structure, bacterial levan shares many of the characteristic solution properties of disordered linear polysaccharides, including specifically:

(1) typical solution-like response to low-deformation oscillatory shear (Fig. 6);
(2) absence of detectable conformational change with temperature (Fig. 5);
(3) Cox–Merz superposition of η and η^* (Fig. 6);
(4) similar shear thinning (Fig. 3);
(5) the onset (Fig. 4) of semi-dilute solution properties at a closely comparable degree of space-occupancy ($c^{**}[\eta] \approx 3{\cdot}6$ for levan; $c_{crit}[\eta] \approx 4$ for linear polysaccharides).

These marked similarities all point to a similar mechanism of intermolecular interaction by physical entanglement rather than by any form of specific non-covalent association. The unusual features of the levan system are:

(1) exceptionally low intrinsic viscosity (i.e. small hydrodynamic volume) for a polymer of high molecular weight;
(2) unusual sensitivity of viscosity to increasing concentration between c^* and c^{**};
(3) extreme concentration-dependence of viscosity ($\sim c^{9{\cdot}3}$) at $c > c^{**}$.

As already discussed, low intrinsic viscosity is an expected consequence of a densely packed, branched structure. The unusually steep concentration-dependence of solution viscosity for levan at concentrations above c^* can be rationalised by the reptation model developed by de Gennes and by Doi and Edwards.

Above c_{crit} a single polymer molecule is regarded as lying in a 'virtual tube' made up of the surrounding network of other chains. The easiest way for it to escape is to wriggle along the length of the tube, in the manner of a snake or reptile (hence 'reptation'). The Newtonian viscosity is then related to the relaxation (or disentanglement) time, τ_d, the time for the macromolecule to escape completely from the tube. The tube, being a dynamic arrangement of chains which are themselves moving, also has its own lifetime (De Gennes, 1979; Ferry, 1980).

The behaviour of branched polymers is more complex still (Graessley, 1977). Centrally, however, the steep increase in η_0 for levan at concentrations above the onset of entanglement is an expected consequence of the much greater time required for a branched chain to extract several arms from a branched tube.

Polymer exclusion and phase-separation

The other striking consequence of the densely branched structure of levan is its effectiveness in resisting interpenetration by other polymers, leading to the major reductions in viscosity shown in Figs 7 and 8, and to macroscopic phase-separation. Since, as already discussed, polymer exclusion effects originate in enthalpically unfavourable interactions between unlike polymer segments, a branched polymer, radiating closely spaced chain-segments, will generate far greater resistance to interpenetration than an open coil which can respond by contracting away from the 'foreign' species. Thus dextran is often used to create two-phase liquid systems for separation of solid particles by partitioning between the phases (Albertsson, 1970).

As expected for a polymer-exclusion phenomenon, suppression of viscosity in mixed solutions of levan with plant polysaccharides can be readily diluted out, in contrast to 'synergistic' associations (e.g. xanthan–LBG), which persist to extremely low concentrations. The concentrations of levan surrounding bacterial cells, or microcolonies of cells *in vivo*, however, are substantially higher than those used in our solution studies of interactions *in vitro*. It seems reasonable to conclude, therefore, that the same major exclusion effects will occur when levan comes into contact with $(1 \rightarrow 4)$-linked polysaccharides at the inner surface of leaf mesophyll tissue during bacterial invasion, and, as discussed below, could be of considerable physiological significance.

Levan in pathogenesis

One of the principal defence reactions of plants against bacterial attack is the 'hypersensitive response'

(Klement, 1982) which occurs when an individual plant cell is killed by the invading pathogen. Collapse of the plant cell membrane releases a flood of bactereostatic compounds and oxidation products, giving protection against further attack over a wide area around the 'sacrificial' cell. It has been demonstrated that initiation of the hypersensitive response requires intimate morphological contact between the cells of host and parasite (Stall & Cook, 1979). When this morphological contact was inhibited by enveloping the bacteria with agar, or by artificially maintaining the intercellular spaces of the mesophyll in a water-soaked state, a compatible interaction (i.e. disease) occurred even with incompatible pathogens.

The results of our present work suggest that levan, because of its extreme resistance to interpenetration by plant polysaccharides, may be particularly effective in preventing morphological contact, thus allowing bacteria embedded in their protective coating of levan-rich slime to continue to live and propagate. Moreover, levansucrase, being a sturdy exoenzyme, could precede the local bacterial attack, and utilise the translocatory sucrose of the host to create a protective levan shield in advance of colonisation by the pathogen. It has been demonstrated (Gross & Kindl, unpublished), that neither levan nor alginate (the other main polysaccharide from *Pseudomonas phaseolicola*) elicit a hypersensitive response in batch-grown cell cultures of *Arachis hypogea*, even at concentrations up to 1 mg ml^{-1}.

It is likely that other features of the levan–levansucrase system may act synergistically with the barrier function proposed above. Specifically, it may

(1) deprive the host of nutrients by assimilation of sucrose, simultaneously providing glucose as a substrate for the bacteria;
(2) create local sinks at infection sites, thereby disrupting the natural flow of assimilates;
(3) interrupt the local apoplasmatic supply of carbohydrate to mesophyll cells by converting sucrose to a form no longer accessible to the host;
(4) inhibit diffusion of gases to the slime-enveloped bacterial cells, thus partially offsetting the unfavourably high oxygen tension within plant tissue.

There are certainly also additional effects on the long-term survival of the bacteria and their fitness for dispersal (Bauske, 1967).

All the above effects, however, show up only in the late stages of the disease. We suggest, therefore, that the primary function of bacterial levan during pathogenesis is to block the host–pathogen recognition (i.e. the morphological contact between the cell walls of host and parasite) by forming an excluded layer at the surface of the plant cells. If this proposal is correct, the levan–levansucrase system would clearly merit substantial further study as an important factor in controlling the virulence of many plant pathogenic bacteria.

ACKNOWLEDGEMENTS

We thank Mrs U. Hettwer for communication of unpublished results from her PhD research and for participation in the studies of viscous interactions reported here. We also thank Prof. S.B. Ross-Murphy for helpful discussions and advice.

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