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# Exopolysaccharides from *Rhizobium meliloti* YE-2 grown under different osmolarity conditions: viscoelastic properties

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## ABSTRACT

The exopolysaccharides from *Rhizobium meliloti* YE-2 extracted 10 days after the inoculum from culture broths and having different osmolarity values (0, 0.2, 0.4, and 0.6M) have been investigated by means of oscillatory and steady-shear measurements on 1% solutions in 0.1M NaCl. The micro-organism produces a mixture of a galactoglucan and a succinoglycan. At low osmolarity (0 and 0.2M), the main fraction consists of galactoglucan and the viscoelastic properties of the mixture are typical of an entanglement system. At 0.4 and 0.6M, the proportion of the succinoglycan increases and the viscoelastic properties change abruptly to those typical of a "weak gel" system. Different conformations are adopted by the two exopolysaccharides in solution.

## INTRODUCTION

In the last decade, increasing attention has been paid to the exopolysaccharides produced by fast-growing *Rhizobium* strains. On the one hand, *Rhizobia* participate in the nitrogen-fixing, symbiotic relationship with host legumes, and their exopolysaccharides seem to play an important role in defining host specificity[1]. On the other hand, the ability of the polysaccharides to give highly viscous aqueous solutions, even at low concentrations, has been considered very attractive for potential applications[2].

The *Rhizobia* strains belong to the group of *Agrobacterium*. *R. meliloti* usually produces a high-molecular-weight succinoglycan-type exopolysaccharide[3,4] with the composition glucose:galactose:pyruvate:succinate:acetate 7:1:1:1:1. The solution properties of the succinoglycan from *R. meliloti* M5N1 have been studied[2].

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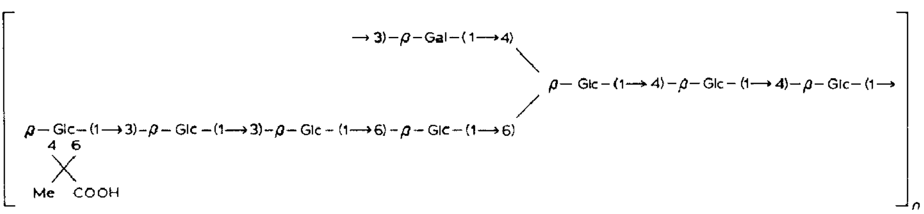
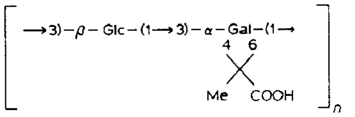
Mutant (variant) strains also exist with different patterns of production of exopolysaccharides. The slimy variant strain YE-2(S1) of *R. meliloti* produces two different water-soluble exopolysaccharides that are not amenable to ethanol fractionation[3,5]. In addition to the usual succinoglycan (EPS1), a galactoglucan (EPS2) is produced with the composition glucose:galactose:pyruvate:acetate 1:1:1:1. Such a galactoglucan has been found as the high-molecular-weight exocellular product of *Achromobacter* spp. and *Agrobacterium radiobacter*[3]. The proposed structures[5] of the succinoglycan (**1**) and the galactoglucan (**2**) are as shown. A common pathway of biosynthesis has also been proposed for the two exopolysaccharides[3].



**1** (EPS1 plus succinyl and acetyl groups)



**2** (EPS2 plus acetyl groups)

The osmotic conditions of the culture medium markedly affect the pattern of production of the EPS[5]. At low osmolarities (no added NaCl), the mixture of EPSs comprise ~75% of galactoglucan and ~25% of succinoglycan. On increasing the osmolarity of the medium[5], the proportion of succinoglycan increases up to 85% in a culture medium with 0.6M added NaCl. Such a change affects the viscosity properties of the culture media[5] and we now report further on this phenomenon.

## EXPERIMENTAL

*Materials.* — *R. meliloti*, slimy variant strain YE-2(S1), was grown at the Department of Microbiology, Agricultural University, Wageningen, The Netherlands, as described[5]. The samples of the glycan mixtures, kindly provided by Professor L. P. T. M. Zevenhuizen, were recovered 10 days after the inoculum from culture broths having different osmolarity values (0, 0.2, 0.4, and 0.6M NaCl) and are from the same batch used in a study[5] of the influence of osmotic variation on EPS production. The samples are designated 00-10d, 02-10d, 04-10d, and 06-10d, respectively (Table I). Aqueous 1% solutions of the exopolysaccharides were prepared by prolonged stirring of the freeze-dried polymers in 0.1M NaCl at room temperature, using screw-top bottles.

TABLE I

Data for EPS1 and EPS2[a]

| *Osmolarity (M NaCl)* | *Weight fraction* | | *Mole fraction* | | $[\eta]$ |
|---|---|---|---|---|---|
| | *EPS1* | *EPS2* | *EPS1* | *EPS2* | |
| 0.0 | 0.25 | 0.75 | 0.14 | 0.86 | 5.4 |
| 0.2 | 0.50 | 0.50 | 0.27 | 0.73 | 5.1 |
| 0.4 | 0.78 | 0.22 | 0.56 | 0.44 | 17.0 |
| 0.6 | 0.85 | 0.15 | 0.69 | 0.31 | - |

[a] The molecular weight of the repeating units of the EPS1 and EPS2 are 1508 and 436, respectively.

*Methods.* — Oscillatory and steady-shear measurements were carried out at 25°, using a Rheometrics Mechanical Spectrometer RMS-605 with automatic computation and plotting of the results. The following cone and plate geometry was used: diameter, 50mm; cone angle, 0.04 radian. Steady-shear measurements in the range $10^{-1}$ to $10^3$ $s^{-1}$ and dynamic oscillatory measurements in the range $10^{-2}$ to $10^2$ rad. $s^{-1}$ were made using the high-sensitivity transducer ST-10. Evaporation of the solvent was prevented by a layer of low-viscosity silicone oil (Dow Corning 200/10cs). All measurements were carried out within the established linear viscoelastic regime.

## RESULTS AND DISCUSSION

*Characterisation of the systems.* — Increase in the osmolarity affects the rheological properties of the culture media[5] by modifying markedly the pattern of production of EPS by *R. meliloti* YE-2(S1).

The two exopolysaccharides produced have different structures and sugar compositions; in particular, the ratio of acidic groups to total sugar units changes from 1:2 to 2:8 for EPS1 and EPS2, respectively. Thus, the two EPSs would exhibit different polyelectrolyte character, at least under conditions of low ionic strength. Therefore, all measurements were carried out on solutions in 0.1M NaCl. Furthermore, as a consequence of the different structure of the two EPSs, a change would be expected in the properties due solely to the way in which the composition of the mixture is expressed, *i.e.*, mole/L (spectroscopic or thermodynamic studies) or g/L (viscosity measurements). The viscosity data, shown in Table I, indicate that, as far as the viscosity of the mixture (even in the infinite dilution $[\eta]$ limit) is concerned, the EPSs are combined non-additively. Consequently, the osmolarity-induced change from galactoglucan to succinoglycan synthesis should be reflected in the viscoelastic properties of the mixtures.

*Dynamic oscillatory shear experiments.* — The viscoelastic spectrum of the mixture isolated from the culture broth in the absence of NaCl is reported in Fig. 1A. The viscoelastic behaviour is typical of a dilute entanglement system[6]. In the range of frequencies investigated, the response is mainly liquid-like with $G'' > G'$. Both $G'$ and $G''$ are strongly dependent on frequency, but $G'$ rises faster than $G''$ with $\omega$ and the two

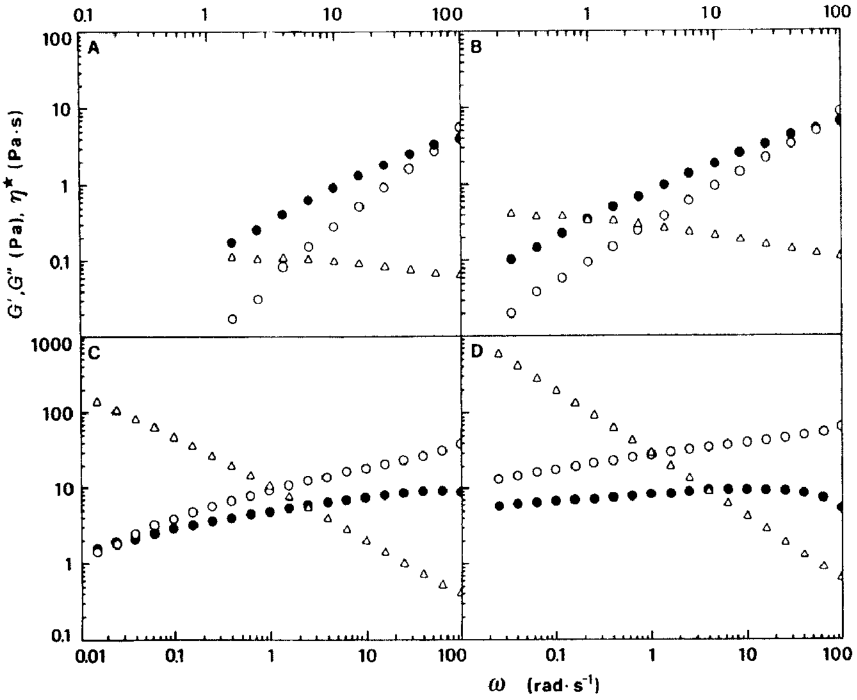


Fig. 1. Frequency sweeps ("mechanical spectra") for 1% solutions of 0-10d (A), 02-10d (B), 04-10d (C), and 06-10d (D) in 0.1M NaCl. (○) $G'$; (●) $G''G''$; (△) $\eta^*$.

curves eventually intersect at a "cross-over" frequency of ~80 rad.s$^{-1}$. The maximum frequency dependence is approximately $G' \propto \omega^{1.5}$ and $G'' \propto \omega^{0.85}$, and is in fairly good agreement with the expected frequency dependence of a liquid system with $G' \propto \omega^2$ and $G'' \propto \omega^1$. The complex viscosity, $\eta^* = (1/\omega)[(G')^2 + (G'')^2]^{0.5}$, shows a tendency towards frequency independence at low frequencies; on increasing the oscillatory frequency, $\eta^*$ decreases and assumes an approximate power-law dependence.

The mixture of EPSs isolated from the medium with 0.2M osmolarity shows similar viscoelastic behaviour (Fig. 1B). However, the frequency dependence of both $G'$ and $G''$, especially at low frequency, shows deviations from that of a typical entanglement system with $G' \propto \omega^{0.9}$ and $G'' \propto \omega^{0.8}$, and the cross-over frequency shifts to the lower value of ~60 rad.s$^{-1}$.

On increasing the osmolarity to ≥0.4M, the viscoelastic behaviour of the mixture of EPSs changes abruptly, as shown in Figs. 1C and 1D. Fig. 1C shows that, at high frequency, the $G'$ values are significantly greater than the $G''$ values and are only slightly frequency dependent. At lower frequencies, the curves of $G'$ and $G''$ approach and cross at a frequency of 0.016 rad.s$^{-1}$. The complex viscosity is strongly frequency dependent in the whole range of frequencies investigated. The viscoelastic behaviour of the 06-10d sample is distinct from that of an entanglement system and is essentially the behaviour of a "weak gel" system (see Fig. 1D), with only very small frequency dependence of both $G'$ and $G''$. The complex viscosity decreases markedly with increasing frequency dependence (log–log slope ~0.8).

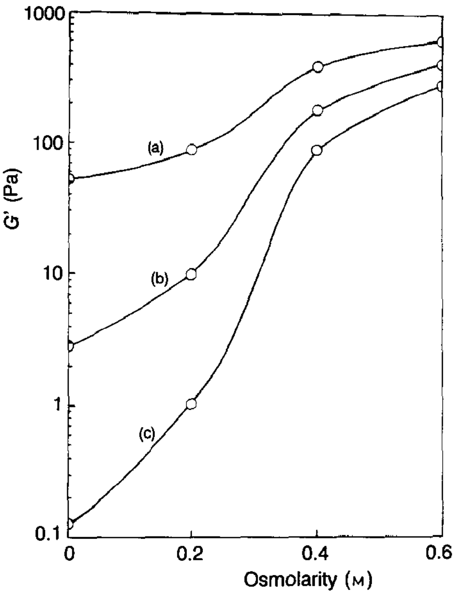


Fig. 2. Storage modulus $G'$ at (*a*) 100, (*b*) 10, and (*c*) 1 rad/s, as a function of the osmolarity of the culture broth of *R. meliloti* YE-2.

The osmolarity-induced change from galactoglucan to succinoglycan biosynthesis is shown in Fig. 2, where $G'$ (on a log scale) is plotted at three different frequency values. The storage modulus is increased dramatically on passing from the 02-10d to the 04-10d sample and this change is more evident at low frequency values.

The increase of the osmolarity of the culture medium produces an increase in the proportion of succinoglycan, and the viscoelastic spectrum of the mixture containing the largest proportion (85%) of the succinoglycan is typical of a conformationally ordered polysaccharide (at sufficiently high concentration and molecular weight) that can form a "weak gel" system through specific intermolecular interactions[7]. This situation is found, for example, for xanthan, produced by *Xanthomonas campestris*[7-10], wellan from *Alcaligenes* (ATC 31555)[11], and the EPS from *Bacillus polymyxa* (NCIB 11429)[12].

*Steady-shear experiments.* — The shear-rate-dependent behaviour of the samples 00-10d and 02-10d is shear thinning as shown in Fig. 3A for the 00-10d sample. At low shear rates, an (upper) Newtonian plateau is evident and both the zero-shear-rate viscosity and the shear-rate value at which the onset of shear-thinning behaviour occurs, can be evaluated. The former are 0.12 and 0.40 Pa s, respectively, and the onset of shear thinning occurs at shear rates of ~ 10 and ~0.7 $s^{-1}$, respectively.

The frequency dependence of $\eta^*$ and the shear-rate dependence of $\eta$ for many disordered polysaccharides are closely superimposable[13] and this, originally semi-empirical correlation, is known as the Cox–Merz rule[14]. Samples 00-10d and 02-10d follow this rule (see Fig. 3A) and, in the whole range of frequencies and shear rates investigated, the curve of $\eta^*(\omega)$ is essentially coincident with that of $\eta(\dot{\gamma})$.

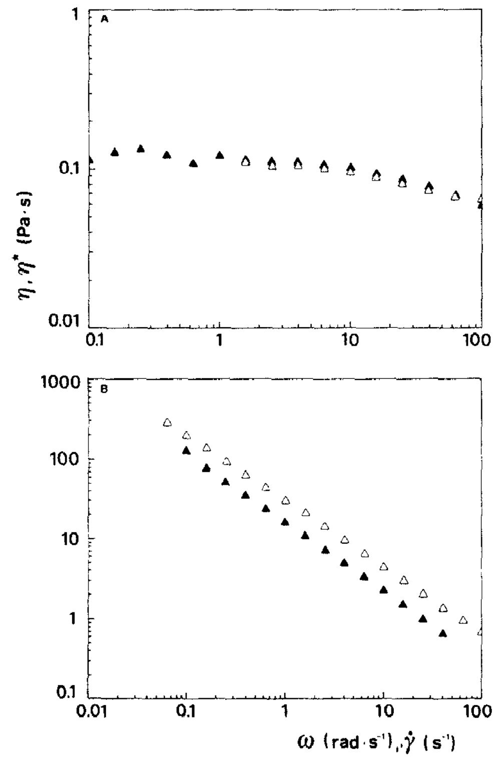


Fig. 3. Cox–Merz plot for 1% solutions of 0-10d (A) and 06-10d (B) samples in 0.1M NaCl. (△) $\eta^*(\omega)$; (▲) $\eta(\dot{\gamma})$.

The rheological behaviour of samples 04-10d and 06-10d is also shear thinning; however, the profiles of the flow curves (log $\eta$ *vs.* log $\dot{\gamma}$ plots) change dramatically. The steady-shear viscosity is strongly shear-rate dependent, and exhibits an approximate power-law behaviour with $\dot{\gamma}$ over the range of shear rate investigated (the exponents are ~0.8 and ~0.9, respectively) with no evidence of the upper Newtonian plateau, as shown in Fig. 3B for 06-10d. Moreover, the Cox–Merz rule fails over the whole range of frequencies and shear rates investigated, *i.e.* the $\eta^*(\omega)$ and $\eta(\dot{\gamma})$ curves are not superimposable (see Fig. 3B).

Some of the quantitative changes in rheological properties noted above are, at least superficially, consistent simply with an increase in the mol. wt. of the EPS as the osmolarity of the culture media is increased. For example, this increase would produce, at a given concentration of EPS, a number of effects, *viz.* a shift of the cross-over frequency to lower frequencies, a plateau in $G'$ at the higher frequencies, a higher zero-shear Newtonian viscosity, and a shear-thinning power-law regime extended to lower frequencies and shear rates. However, the difference between entanglement networks and weak gels (or "structured liquids") is as much qualitative as quantitative, and, as outlined above, two of the clearest indications of this difference are the failure of the Cox–Merz rule and the power-law exponents > ~0.8 in steady-shear experiments.

Together, these factors are diagnostic of such systems, *i.e.* those in which, in addition to physical entanglements, more specific intermolecular interactions between ordered polymer chains appear to be involved[7,15].

*Conformations in solution.* — An order–disorder temperature-induced conformational transition has been observed[2] by means of optical rotation and viscosity measurements for the succinoglycan produced by *R. meliloti* M5N12. In particular, in dilute (0.1%) aqueous solution and at high ionic strength (0.1M NaCl), the phenomenon is accompanied by a remarkable decrease in relative viscosity at 76° ($T_m$). A similar conformational transition has been observed[16,17] for the succinoglycan produced by *Pseudomonas* sp NCIB 11592, for which the ordered conformation is also favoured at high ionic strength, and both optical rotation and viscosity change abruptly at a $T_m$ that reaches 72° at M NaCl. This transition is consistent with a stretched-helix to flexible chain (or coil) transformation.

Although different microbial strains and culture conditions have been used, the primary structures of these EPSs are identical to that of the succinoglycan (**1**) (notwithstanding the content and location of "minor" substituents). Accordingly, it is reasonable to attribute the weak-gel viscoelastic behaviour of the 06-10d mixture to the ordered conformation adopted by the succinoglycan in 0.1M NaCl (*i.e.*, at high ionic strength) at 25°. The ordered-chain conformation is the major contributor in stabilising rheologically effective intermolecular interactions. The tetrasaccharide side-chain of the succinoglycan may play a crucial role in promoting the formation of ordered structures[11], as observed for the succinoglycan from *Pseudomonas.*

In contrast, the overall rheological behaviour of the galactoglucan-rich mixture is that of a typical entanglement system. This behaviour is exhibited by such flexible random-coil polysaccharides as guar, a galactomannan obtained from the seed endosperm of certain legumes[15,18]. This observation suggests that, in solution, a disordered conformation is adopted by the galactoglucan, an observation consistent with the preliminary experimental results of the conformational characterisation in dilute solution[19].

The primary structure (**2**) of the galactoglucan involves alternating (1→3)-linked β-D-Glc and α-D-Gal. A (1→3)-β-D-glucan has a flexible and loosely-helical conformation[20] with a characteristic ratio $C_\infty$ of ~ 3, a value lower than that (~ 5) of amylose[21]. On the other hand, a (1→3)-α-D-galactan has an extended ribbon-like conformation[20] with a $C_\infty$ value of 30. The $C_\infty$ value of ~ 30 for the galactoglucan is consistent with a more rigid random-coil conformation. The presence of the pyruvate residue, under certain conditions of pH and/or ionic strength, may modify or restrict the conformational options of the backbone of the galactoglucan. Elongation of the chain is also revealed by X-ray diffraction patterns[22] which indicate that the polysaccharide chain exists as a 2-fold single helix with a pitch of ~ 16 Å.

With such a hypothesis, it must be assumed that, at intermediate osmolarities, the presence of the more flexible and disordered galactoglucan chains reduces the possibility for succinoglycan chains to form weak associations, and consequently the viscoelastic response results are intermediate of those of an entangled system and a weak gel. In fact, the viscoelastic behaviour ("mechanical spectra") of the mixture recovered from a culture broth of osmolarity 0.4M is remarkably similar to that obtained[23,24] for xanthan/guar blends. It is possible that the change in the pattern of EPSs in response to variation of the external conditions may be to create a "more congenial" environment for the microbial cells.

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