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Chemical and rheological properties of bacterial succinoglycan with distinct structural characteristics

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ABSTRACT

Succinoglycans are bacterial exopolysaccharides with an octasaccharide repeating unit, composed of glucose and galactose in a 7:1 molar ratio of, and non-carbohydrate substituents, including pyruvate, succinate and acetate. The succinoglycans produced by four different strains of *Sinorhizobium meliloti*, gram-negative soil bacteria, were analyzed for their molecular weight distribution and degree of non-carbohydrate substitution, as well as their chemical properties were related to their rheological properties. These results showed that the ratio of high molecular weight to low molecular weight succinoglycan was varied from 0.50 to 2.36. Degree of succinylation among the bacterial strains was in the range of 0.30–1.90. Therefore, we concluded that each strain produced succinoglycans with different average degrees of polymerization and succinylation; and that these characteristics were correlated to the rheological properties of the solutions. The effect of molecular weight on the rheological properties appeared to be less than that of the succinyl group abundance.

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1. Introduction

An extracellular polysaccharide, succinoglycan, is produced by *Sinorhizobium, Agrobacterium* and other soil bacteria. Succinoglycan molecules with relatively high molecular weight produce viscous solutions (Zevenhuizen, 1997). This, along with acid stability makes succinoglycans an attractive thickening agent for the food and commodities industries. The succinoglycans repeating unit is a branched octasaccharide of seven β-linked glucose (Glc) residues and one β-linked galactose (Gal) residue, with non-carbohydrate substitutions of acetate on the backbone, and pyruvate and succinate on the branch (Fig. 1). *Sinorhizobium meliloti* produces two distinct size ranges of succinoglycan, high molecular weight (HMW; >100,000 Da) and low molecular weight (LMW; <5000 Da), and the specific size range HMW/LMW ratio vary depending on the source (Ridout, Brownsey, York, Walker, & Morris, 1997;Simsek, Ojanen-Reuhs, Stephens, & Reuhs, 2007). Also, the degree of the non-carbohydrate substitutions is dependent upon the bacterial source. Fig. 1 shows the structure of a succinoglycan obtained from *S. meliloti* with the possible succinylation sites indicated by A and B.

The characteristics of succinoglycans in aqueous solutions have been analyzed by several researchers (Balnois et al., 2000; Gravanis, Milas, Rinaudo, & Tinland, 1987; Kaneda & Yanaki, 2002; Kido, Nakanishi, Norisuye, Kaneda, & Yanaki, 2001). It shows reversible pseudoplastic behavior in solution (Gravanis, Milas, Rinaudo, & Clarkesturman, 1990; Ridout et al., 1997). The bacterial origin of the polysaccharide and the degree and type of non-carbohydrate substituents determine the rheological properties of succinoglycan solutions (Cesaro, Gamini, & Navarini, 1992b; Gravanis et al., 1987). In solution, succinoglycans exhibit a temperature dependent sharp order–disorder transition, which has been examined by ^{1}H NMR (Gravanis et al., 1990), differential scanning calorimetry (DSC) (Boutebba, Milas, & Rinaudo, 1997), optical rotation (OR), conductivity measurements and viscosity studies (Cesaro et al., 1992a; Dentini, Crescenzi, Fidanza, & Coviello, 1989; Fidanza, Dentini, Crescenzi, & Delvecchio, 1989; Gravanis et al., 1987; Gravanis et al., 1990). When temperature or ionic strength increases, ordered forms become dominant. The temperature-and ionic-strength-dependent differentiation of transition is an expected property, as in a helix-coil transition. The viscosity of succinoglycan samples decreases with heating it to above the transition temperature (Dentini et al., 1989; Gravanis et al., 1990). An irreversible decrease in molecular weight follows this change in viscosity, and chain breakage or disruption of aggregates can also cause these differences (Fidanza et al., 1989; Gravanis et al., 1987). The observation of somewhat aggregated single helices in aqueous solutions supports this concept. When the chemical charged groups are removed, stiffening occurs in the structure of polymer because of the stabilization of the helical backbone.

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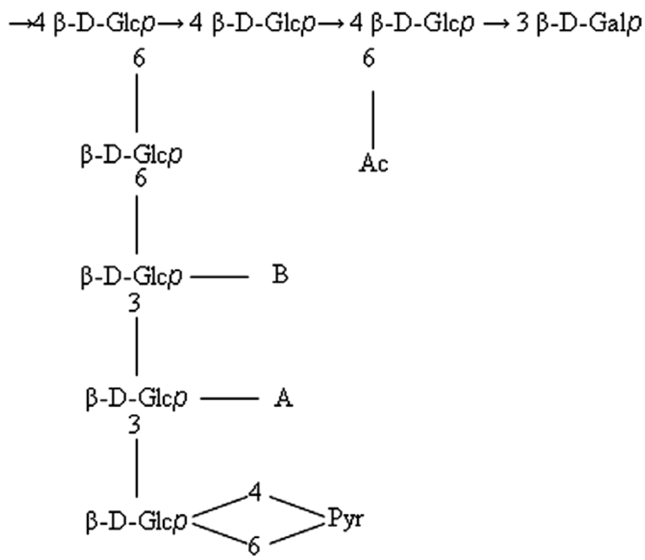

Fig. 1. Repeating unit of Succinoglycan. The possible substitution for succinate is shown with A and B. A can carry a succinate substitution; B may only carry a succinate if one is present on A.

Although, past studies have focused on the relation between the chemical structure of succinoglycans and rheological properties of their liquid suspensions measured under steady state conditions, notably viscosity, less work has been performed in the characterization of viscoelastic properties of gel systems formed with these succinoglycans. Viscoelastic characterization of succinoglycans using oscillatory dynamic testing has several advantages. Firstly, the testing is done at deformations low enough so that the measurements do not disturb the sample. Secondly, viscoelastic properties of samples obtained from small deformation oscillatory dynamic testing may provide a macroscopic representation of the sample chemical and structural make-up. Lastly, potential applications of the succinoglycans in food and non-foods products are related to functional properties of these systems associated with their gelling capacity. In this study, the succinoglycans produced by four different *S. melliloti* strains has been chemically and rheologically characterized and their chemical properties related to their rheological properties.

2. Materials and methods

2.1. Bacterial strains, growth conditions and production of succinoglycan

Four different *S. meliloti* strains, NRG185, NRG247, NRG34 and Rm41, were used in this study. Cells were grown in a glutamate-mannitol-salts (GMS) medium (pH 7.0) supplemented with biotin, thiamine, and trace elements as described by Zevenhuizen and Van Neerven (1983). One liter of GMS medium in a 2-liter Erlenmeyer flask was inoculated with 10 ml of overnight culture and growth at 28 °C for 5 days with shaking (120 rpm). Cells were centrifuged at 20,000*g* for 30 min, and the clear culture supernatant that contains succinoglycan was concentrated by roto-evaporation and then dialyzed against water for 2 days at 4 °C, with several changes of water. It was again concentrated and freeze dried.

2.2. Size exclusion chromatography (SEC) analysis

SEC analysis was performed with a Dionex BioLC chromatographic system (Dionex Corp, Sunnyvale, CA) equipped with a Hewlett-Packard 1047A (Hewlett-Packard Company, Palo Alto, CA) refractive index detector, and Superose™ 12 column (Amersham Bioscience, Piscataway, NJ). The sample (500 μg) was dissolved in 0.5 ml of ammonium formate buffer (50 mM; pH 5.5), injected into the system, and eluted with same buffer at a flow rate of 0.4 ml/min. Integration was performed to determine the ratio of high molecular weight (HMW) to low molecular weight (LMW) succinoglycans.

2.3. Proton nuclear magnetic resonance (^1H NMR) spectroscopy

For ^1H NMR Spectroscopy the samples were dissolved in 1 ml deuterium oxide (D_2O), freeze dried and again dissolved in 0.6 ml D_2O. ^1H NMR spectra were obtained at 25 °C using a Varians UNITY INOVA 300 NMR spectrometer (Varian Inc, Palo Alto, CA).

2.4. Rheological analysis

The rheological properties were determined using a rheometer (AR2000, from TA Instruments). The parallel plate geometry was used and strain sweep tests were conducted to determine the linear viscoelastic range of the samples. Oscillatory dynamic tests were carried out by controlling the applied strain (within the linear viscoelastic range) at varying frequencies in a range 3–63 rad/s. In addition to the viscoelastic properties, flow curves of the samples were also obtained using a cone and plate geometry (2°–40 mm diameter cone) in a range of shear rates of 1–30 1/s.

3. Results

3.1. Analysis of succinoglycans from different S. meliloti strains by SEC and ^1H NMR

Extracted and purified succinoglycan samples from the four different *S. meliloti* strains were analyzed by SEC and ^1H NMR to determine chemical properties. Since molecular weight distribution plays an important role for the functional properties of a polysaccharide, samples were analyzed by SEC to determine the ratio of HMW to LMW succinoglycans (Fig. 2). The results are summarized in Table 1. These results showed the following HMW/LMW ratios for each strain: *S. meliloti* Rm41, 2.36; *S. meliloti* NRG185, 0.50; *S. meliloti* NRG34, 0.91; *S. meliloti* NRG247, 1.56.

^1H NMR was performed to determine the degree of substitution of these samples and results are shown in Fig. 3. These results were used to determine the degree of non-carbohydrate substitution by integration of the relevant resonances; the values are reported in Table 1. Degree of pyruvate was assumed the same for all the samples, which is one, and the degree of acetylation slightly varied among the samples (Simsek et al., 2007). However, the amount of succinate in each sample was significantly different (Table 1). *Sinorhizobium meliloti* Rm41 had the lowest degree of succinylation of 0.30, *S. meliloti* NRG185 the largest with a value of 1.90, whereas, for *S. meliloti* NRG34 and *S. meliloti* NRG247 succinate degrees were 0.76 and 1.81, respectively.

3.2. Analysis of rheological properties

The viscoelastic properties of succinoglycan solutions are shown in Fig. 4. Prior to the rheological characterization, 1% of the freeze dried succinoglycan samples were dissolved in purified water and stored for at least 24 h to ensure full hydration. As shown in Fig. 4, at low frequencies, viscous modulus values (G'') were much larger than the elastic modulus values (G'), and hence a liquid-like behavior predominated for all the samples. However, as the frequency increased the elastic modulus took over, which is indicative of a more elastic behavior of these solutions prevailing at high frequencies. Solutions of the succinoglycan samples ob-

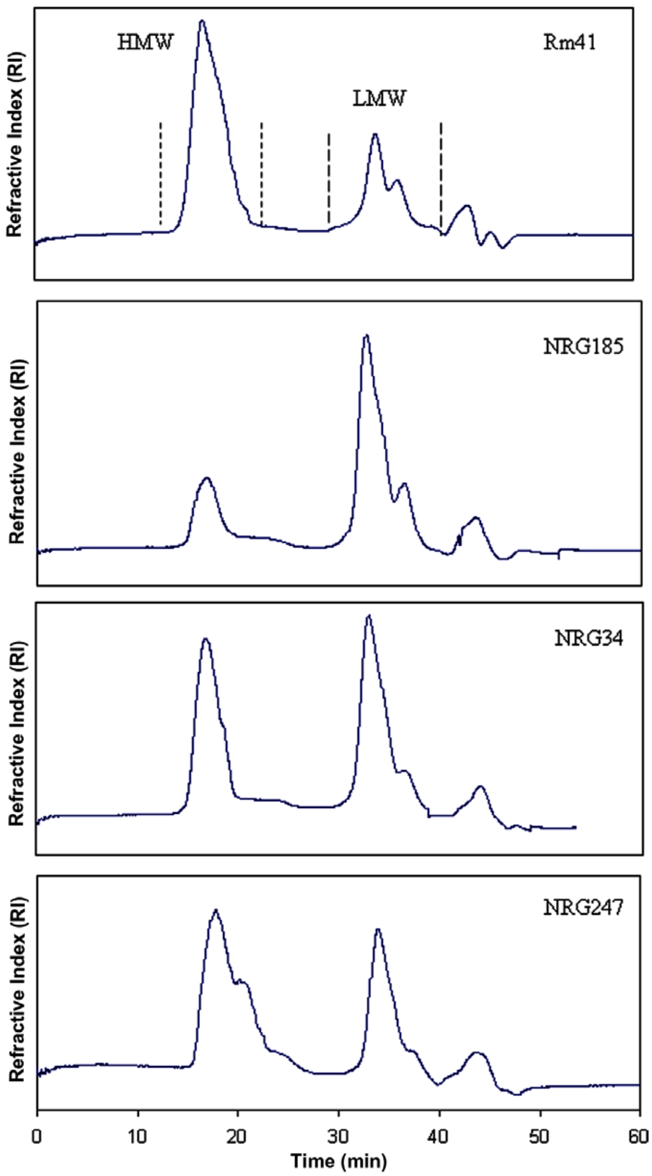

Fig. 2. Size exclusion chromatography analysis of succinoglycan from *S. meliloti* strains. High molecular weight (HMW) and low molecular weight (LMW) succinoglycan fractions were labeled at the top of the figure.

Table 1
The high molecular weight (HMW)/low molecular weight (LMW) ratio and degree of non-carbohydrate substitution of bacterial succinoglycans.

Sinorhizobium meliloti strain	Molar ratio of substituents (substituent/repeat)			
	Pyruvate[a]	Acetate[a]	Succinate[a]	HMW/LMW ratio[b]
Rm41	1.00	0.79	0.30	2.36
NRG185	1.00	0.94	1.90	0.50
NRG34	1.00	0.76	0.76	0.91
NRG247	1.00	0.82	1.81	1.56

[a] Determined by integration of diagnostic resonances in ^{1}H NMR analyses for *S. meliloti* strains (Fig. 3).
[b] Determined by integration of specific peaks from SEC analysis (Fig. 2).

tained from different natural strains showed different cross-over frequencies (frequency at which $G'' = G'$), indicating various degrees of entanglement of these molecules. Typical cross-over frequencies for Rm41, NRG34, NRG247, and NRG185 strains were obtained as 16, 29, 48, and 48 rad/s, respectively. These results

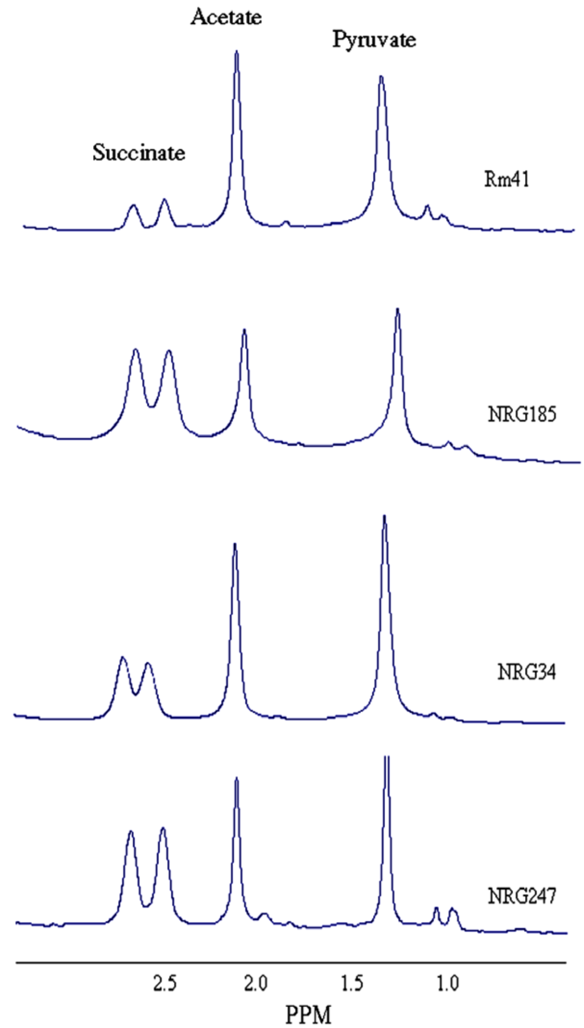

Fig. 3. ^{1}H NMR analysis succinoglycan from *S. meliloti* strains. The diagnostic resonances are labeled at the top of the figure.

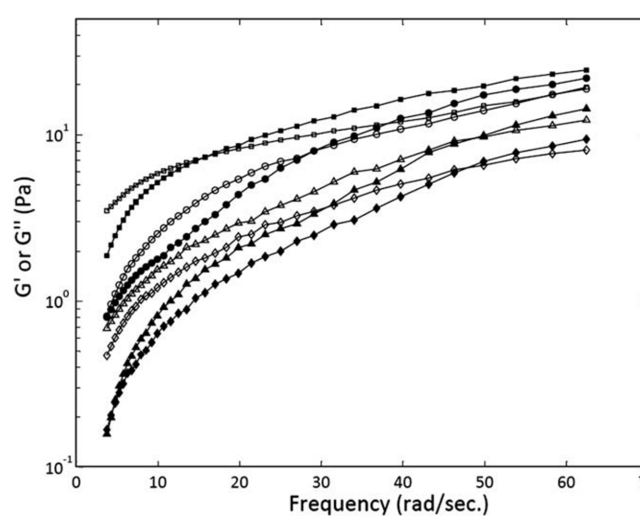

Fig. 4. Viscoelastic parameters of succinoglycan solutions obtained from *S. meliloti* strains. The symbols □, O, Δ, and ◇ represent Rm41, NRG34, NRG247, and NRG185, respectively. Solid symbols indicate storage modulus G' and open symbols indicate G''.

clearly show that the Rm41 sample exhibited a significant higher elastic behavior as a consequence of potentially level of molecular entanglement. Furthermore, the cross-over frequencies were inversely proportional to the actual magnitude of the both viscous and elastic moduli values. The lower the cross-over frequency, the higher the magnitude of the elastic and viscous moduli was determined. Thus, by comparing these results with the chemical analysis of the different succinoglycans it was shown that the differences in viscoelastic properties among the samples can be attributed to the presence of charged substituents such as pyruvate, acetate or succinate. Studies conducted with chemically modified succinoglycans and succinoglycans obtained from genetically modified bacteria revealed that the presence of the succinyl group had an effect significantly larger than that observed with other charged substituents (Ridout et al., 1997). Similar results were obtained in this study, as Rm41 produces succinoglycan with the least succinyl groups and the solutions had the highest elastic and viscous modulus values, compared to those polysaccharides produced by the other strains. It may be that the relatively higher molecular weight of Rm41 succinoglycan is one of the causes of the observed higher modulus values, but comparison of the complex modulus values $\sqrt{G'^2 + G''^2}$ for NRG34 and NRG247 succinoglycans revealed that the absence of the succinyl group had a more profound effect of molecular entanglement and, therefore, moduli values. The flow curves for the different succinoglycan solutions are given in Fig. 5. All samples displayed typical shear thinning behavior following a power law model $\tau = K\gamma^n$. The related power law model parameters are given in Table 2. One percent succinoglycan solutions of Rm41 samples had the highest consistency index (K) and the lowest flow behavior index value (n), which is indicative of a large level of entanglement easily broken down under the action of shear. In other words, Rm41 samples exhibited a significantly higher shear thinning behavior described by the low values of the flow index. On the other hand, 1% succinoglycan solutions obtained from NRG185 strain displayed the lowest consistency index and highest flow behavior index (n), a behavior closely resembling that of Newtonian fluids. A comparison between Rm41 and NRG185 succinoglycans showed that a low succinyl group abundance and relatively high HMW/LMW ratio produces more viscous solutions. When the flow curves of the samples containing similar succinate amount (NRG185 and NRG247) were compared, relatively little difference was observed in the measured apparent viscosities. However, the higher values in the viscosity of the NRG247 sample could be attributed to its higher HMW/LMW ratio. The comparison of the flow curves for the NRG34 and NRG247 samples showed that low amount of succinyl group significantly increased the consistency index and lowered the flow index resulting in a more shear thinning behavior. Table 1 clearly shows that the NRG34 sample had a lower HMW/LMW ratio and succinyl group abundance, when compared to NRG247; however, the flow curves in Fig. 5 showed significantly higher viscosity for the NRG34 solutions, which indicates that an absence of succinyl group is one of the major factors in determining the rheological properties of succinoglycan solutions.

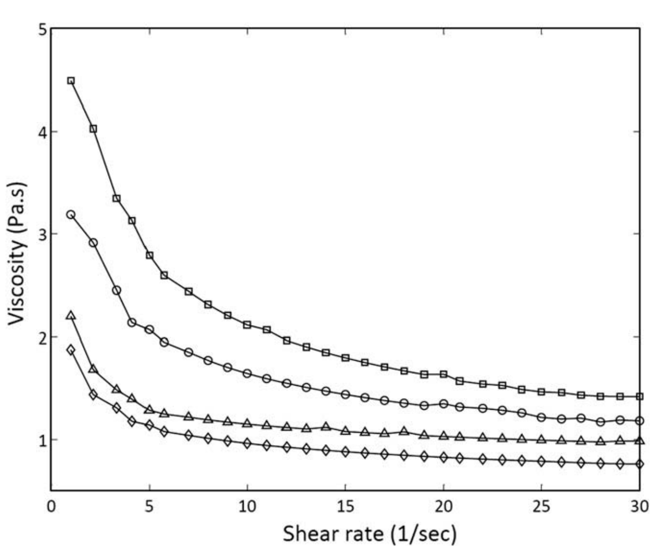

Fig. 5. Flow curves of succinoglycan solutions obtained from *S. meliloti* strains. Symbols are □, O, Δ, and ◇ for Rm41, NRG34, NRG247, and NRG185, respectively.

Table 2
Consistency index (K) and flow behavior index (n) of succinoglycan solutions.

Sinorhizobium meliloti strain	K (Pa.s^n)	n
Rm41	5.05	0.61
NRG34	3.42	0.63
NRG247	1.92	0.79
NRG185	1.79	0.75

4. Discussion

The molecular weight and the nature of the substitution of succinoglycan may vary from source to source. The four strains used in this study were able to produce succinoglycans with different degrees of substitution and molecular weight distributions, as determined by SEC and NMR analyses. When we analyzed the *S. meliloti* NRG34 and NRG247 polysaccharides, succinoglycan from NRG34 showed a lower degree of succinylation than that from NRG247 and a lower ratio of HMW/LMW.

The viscoelastic and flow properties of the succinoglycan samples obtained from four different *S. meliloti* strains were determined. Comparisons of the succinoglycan solutions showed that both molecular weight distribution and the abundance of succinate affect the rheological properties of the solutions. Careful analysis of flow curves and small strain viscoelastic data obtained from small strain oscillatory tests indicate that a low abundance of the succinyl group favors the elasticity and viscosity of the succinoglycan solutions. Succinoglycan samples obtained from different *S. meliloti* strains also differed on molecular weight distribution; however, the effect of molecular weight on the rheological properties appeared to be less than that of the succinyl group abundance.

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