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Viscoelastic properties of aqueous solutions of the phosphopolysaccharide "viilian" from *Lactococcus lactis* subsp. *cremoris* SBT 0495

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Abstract

The viscoelastic properties of the native and partially purified polysaccharide "viilian", excreted by *Lactococcus lactis* subspecies *cremoris* SBT 0495, are studied performing dynamic oscillatory and steady shear measurements. Dynamic as well as steady shear measurements suggest that aqueous solutions of viilian behave as an entangled polymer solution but not as a weak gel. The differences in the viscoelastic spectra obtained in the absence and presence of salt indicates that viilian shows the characteristics of a polyelectrolyte. Addition of 0.1 M NaCl to dilute solutions suppresses the electrostatic interactions giving rise to a more fluid-like character. At higher polysaccharide concentrations involvement of specific interactions between the polysaccharide molecules become apparent, by the increase in the lifetime of entanglement junctions, even when electrostatic interactions are suppressed by the presence of salt. The results are considered in relation to the chemical characteristics of viilian. The viscoelastic properties of viilian most resemble the characteristics of the exocellular polysaccharide from *Cyanospira capsulata*. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Viscoelastic properties; Viilian; *Lactococcus lactis* subspecies *cremoris*

1. Introduction

In ropy fermented milk products such as viili, långfil, and stirred-type yogurt significant amounts of polysaccharides can be found. These polymers are produced by lactic acid bacteria present in the starter cultures used for these products. It is generally believed that these polysaccharides contribute to the stability of the protein gel formed during the acidification of milk (van Marle & Zoon, 1995a), although no direct correlation was observed between the polysaccharide concentration and the viscosity of the fermented milks. From both rheological and microscopic studies it appeared that the polysaccharides have an indirect effect on the protein gel formed during acidification of milk (Schellhaass & Morris, 1985). This might also explain the observations of Zoon, van Marle, Smith and Kingma (1994) that the effect of polysaccharides depends upon the type of polysaccharide formed rather than on their amount. In other words, non-ropy bacteria may also form exocellular polysaccharides but these polymers do not interact with the casein micelles in the acidified milk. In milks fermented with ropy strains fine and regular networks have been observed by microscopic methods (Teggatz & Morris, 1990; Toba, Nakajima, Tobitani & Adachi, 1990; van Marle & Zoon, 1995b), whereas in milk fermented with non-ropy strains the size of the pores was significantly bigger. Studies with chemically acidified milks indicated that in the absence of both bacteria and polysaccharides the pores and flocs in the acidified milk gel were even bigger (van Marle & Zoon, 1995b). Rheological studies of the different gels revealed that ropy strains formed gels with a higher apparent viscosity and a reduced permeability, probably as a result of the fine network structure (van Marle & Zoon, 1995a). As a result of the stabilization of the protein network, milks fermented with ropy strains are less susceptible to syneresis (Toba et al., 1990). The presence of polysaccharides which have specific interactions with the casein micelles gives a slimy consistency which is characteristic of these fermented milk products. The Scandinavian fermented

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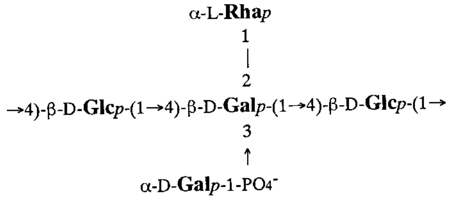

Fig. 1. The structure of the repeating unit of viilian.

milks in particular (e.g., viili, and långfil) have a very sticky appearance which resembles a thick flour dough.

Recently, the structures of some of these polysaccharides have been elucidated (Nakajima, Hirota, Toba, Itoh & Adachi, 1992; Gruter, Leeflang, Kuiper, Kamerling & Vliegenthart, 1992; 1993; Robijn, van den Berg, Haas, Kamerling & Vliegenthart, 1995; Robijn, Thomas, van den Berg, Haas, Kamerling & Vliegenthart, 1995). However, no quantitative data have been published so far on the rheological properties of polysaccharides isolated from the cultures of ropy lactic acid bacteria. Such information is of utmost importance in the elucidation of the mechanisms essential to the stability of fermented milk products, and becomes even crucial in the evaluation of the properties of such polysaccharides in non-dairy foods. The GRAS (Generally Recognized As Safe) status of dairy lactic acid bacteria facilitates the application of their polysaccharides either as additives or as in situ produced thickeners.

Lactococcus lactis subspecies *cremoris* SBT 0495 produces a slime material which contributes to the characteristic sticky consistency of the typical Finnish fermented milk product viili (Nakajima, Toyoda, Itoh, Mukai, Kitazawa & Adachi, 1990; Toba et al., 1990). The slime material consists of polysaccharides and some proteinaceous material. The major polysaccharide in this slime material is a phosphopolysaccharide which is named “viilian” after its original source. Viilian consists of glucose, galactose, rhamnose, and phosphate in a molar ratio of 2:2:1:1 (Nakajima et al., 1992; Oba, Doesburg, & Sikkema, 1998). These components are arranged in repeating units (Fig. 1) which together form the heteropolysaccharide that has an approximate molecular weight of 2 × 10^6 Da (Nakajima et al., 1992). The viilian was isolated and subjected to several rheological measurements in order to characterize its viscoelastic properties. The properties of viilian will be discussed in the light of previous reports on the rheological characteristics of xanthan (Richardson & Ross-Murphy, 1987a), guar gum (Richardson & Ross-Murphy, 1987b), and the exocellular polysaccharide of *Cyanospira capsulata* (CC-EPS) (Navarini, Bertocchi, Cesàro, Lapasin & Crescenzi, 1990). This is the first report on the viscoelastic properties of a purified extracellular polysaccharide from dairy lactic acid bacteria.

Table 1
The polysaccharide content of the samples used for rheological measurements

Sample (%)	Polysaccharide concentration (% (w/v))	
	Water solution	0.1 M NaCl solution
2	—	1.998
1.5	1.501	1.499
1	0.992	0.999
0.75	0.750	0.749
0.5	0.499	0.500
0.4	0.402	0.403
0.3	0.297	—
0.25	0.249	0.249
0.2	0.201	0.198
0.1	0.101	0.099
0.05	0.050	0.049

2. Experimental

2.1. Materials

The phosphopolysaccharide was purified from slime material excreted by *Lactococcus lactis* subspecies *cremoris* SBT 0495 grown batch-wise in a jar-type fermentor on chemically defined medium (Otto, ten Brink, Veldcamp & Konings, 1983) as described previously (Oba, et al., 1998). In the early stationary growth phase, trichloroacetic acid (TCA) was added to a final concentration of 4% (w/v). After 2 h of incubation, the cells and the precipitated proteins were removed by centrifugation (18 000 × *g*, 20 h, 4°C). Slime material was precipitated from the supernatant with ethanol at a final concentration of 60% (v/v). The precipitate from 4 l of culture broth was dissolved in 500 ml ultrapure water (Millipore) and dialyzed against running tap water for 20 h. Subsequently, the dialysis tubes were transferred to a 10 l Erlenmeyer flask and dialyzed five times against 10 l of ultrapure water at 4°C. The water was refreshed every 5 h, except for the third dialysis which was run overnight. After dialysis the slime material was lyophilized and analyzed for its composition. The purity of the polysaccharide was checked by chromatographic analysis on DEAE, and GPC (Nakajima et al., 1990), and by analysis of the chemical composition of the material (Oba et al., 1998). Moreover, the amount of protein present in the sample was determined by a Pierce micro assay.

The lyophilized polysaccharide was dissolved at a concentration of 2.0% (w/v) in ultrapure water. Subsequently, the solution was diluted to the desired concentration (0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 1.5%) and again homogenized. To remove air bubbles from the polysaccharide solutions, all samples were centrifuged at 3000 rpm for 15 min prior to the measurements. In Table 1 the actual weight percentages of the different polysaccharide solutions, as determined from dry weight, are presented.

2.2. Methods

All rheological measurements were carried out at 25°C unless stated otherwise, using a Rheometrics RFS II fluids

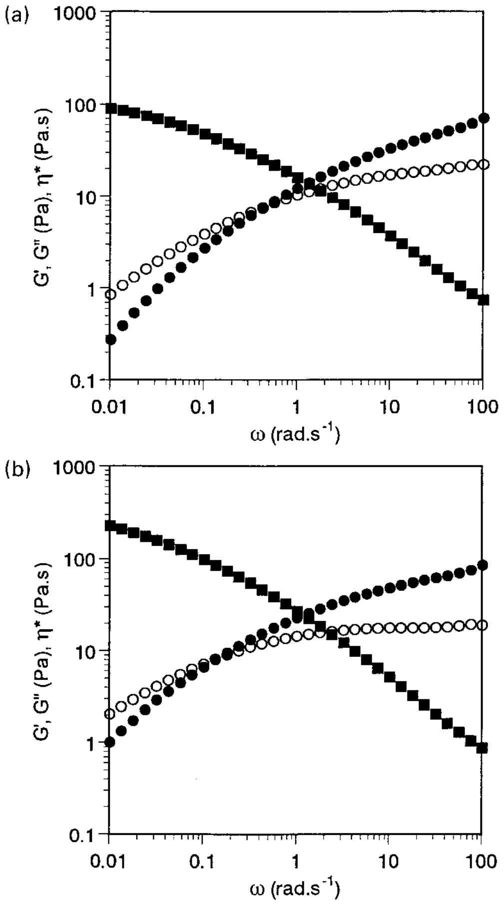

Fig. 2. The frequency sweep for 1.0% (w/v) viilian solutions in water (a) and in 0.1 M NaCl solution (b). Storage modulus, G' (●); loss modulus, G'' (○); dynamic viscosity, η^* (■).

Table 2
The cross-over points ($G' = G''$) at different polysaccharide concentrations (25°C, 1% deformation)

Sample (%)	Water solution		0.1 M NaCl solution	
	$G' = G''$ (Pa)	ω (rad/s)	$G' = G''$ (Pa)	ω (rad/s)
2	—	—	32.0	0.016
1.5	19.9	0.3	17.6	0.042
1	7.02	0.4	8.7	0.14
0.75	4.1	0.91	4.3	0.18
0.5	2.16	3.09	2.01	0.98
0.25	1.06	12.8	0.81	7.1
0.1	0.46	17.8	0.13	22.5
0.05	0.21	21.7	—	—

spectrometer (Rheometrics Inc., Piscataway, USA). The rheometer was equipped with concentric cylinders with diameters of 32 and 34 mm, according to DIN (53-019); the inner cylinder had a cone shaped tip. After filling the cylinder with 10 g of viilian solution, the sample was covered with a thin layer of paraffin oil (Merck, low viscosity grade, Art. 7147) to prevent evaporation. Before the actual measurements, the linear viscoelastic range was determined by a strain sweep test, which indicated linear viscoelastic behavior up to at least 10% deformation at a frequency of 1 rad/s. A strain of 1.0% was used in all our experiments.

3. Results and discussion

3.1. Dynamic oscillatory experiments

The viscoelastic behavior of solutions of viilian is studied by dynamic oscillatory measurements. In Fig. 2, the viscoelastic spectra of the purified viilian solution (1%) both in water and in 0.1 M NaCl are shown. G' and G'' show a continuous increase with ω, the slope becoming steeper as the frequency decreases. This indicates the absence of permanent bonds characteristic of a weak gel. The limiting slopes are not yet attained at the lower end of the applied frequency range (0.01 rad/s) which suggests that terminal relaxation time exceeds 100 s. At high frequencies, a rubbery plateau is approached. This behavior is characteristic of a polymer fluid. At concentrations of 0.05% (w/v) and higher, a clear ‘cross-over’ of the G' and G'' curves can be observed with $G'' > G'$ at low frequencies and $G'' < G'$ at high frequencies (Table 2).

The cross-over frequency ($G' = G''$) marks the time scale at which the transition from liquid-like to fluid-like behavior occurs. At higher frequencies, solid-like behavior dominates, at lower frequency, fluid-like behavior. In the following discussion we will regard this cross-over frequency as a measure of an effective junction lifetime. This does not exclude the existence of junctions of shorter and longer lifetimes. For 1% viilian in water, $G' = G''$ occurs at 0.4 rad/s, which corresponds to an effective junction lifetime of $1/0.4 = 2.5$ s. In 0.1 M NaCl this life time is 7.1 s. Steady share flow experiments did not affect the dynamic data which again points to the absence of permanent bonds.

Comparison with viscoelastic spectra recorded at lower concentrations (data not shown) demonstrates that both G' and G'' increase with increasing polysaccharide concentration, with G' increasing more rapidly than G''. By increasing the concentration of viilian, the cross-over frequency shifts from approximately 22 rad/s (0.05%) to 0.3 rad/s (1.5%) for solutions in pure water and from 22 rad/s (0.1%) to 0.016 rad/s (2.0%) for viilian in 0.1 M NaCl. Effective junction lifetimes increase at higher concentrations as expected.

If we consider the slopes of G' and G'' at the lower end of the applied frequency range, there is an appreciable difference between solutions in pure water and in 0.1 M NaCl (Table 3). The latter show the expected concentration dependence, the limiting slopes of 2 and 1 for the G' and G'' versus ω curves being approached at very low concentrations. Solutions in pure water show a rather scattered concentration dependence for the limiting slopes without a definite trend. This may be due to a polyelectrolyte effect: in salt free systems in vanishing concentration, the polysaccharide chains expand due to intramolecular repulsion

Table 3
The limiting slopes of the storage modulus (G') and the loss modulus (G'') plotted against the frequency determined from the frequency sweep recorded at the different concentrations in water or in 0.1 M NaCl

Sample (%)	Water solution		0.1 M NaCl solution	
	Limiting slope of G'	Limiting slope of G''	Limiting slope of G'	Limiting slope of G''
2	0.91	0.60	0.73	0.48
1.5	0.97	0.62	0.66	0.45
1	1.17	0.73	0.96	0.75
0.75	1.15	0.75	1.04	0.67
0.5	1.13	0.71	1.08	0.81
0.25	0.97	0.78	0.97	0.80
0.1	0.91	0.67	1.64	0.93
0.05	0.86	0.63	1.73	—

between the phosphate groups. At increasing concentration, this effect is counteracted by intermolecular repulsion. In the presence of salt, the polyelectrolyte effect is screened and the expected solution behavior is observed. This also explains the cross-over frequency at very high dilution to be higher in 0.1 M NaCl than in pure water (Table 2). At higher concentrations, the presence of NaCl increases the lifetime of junctions, presumably due to specific intermolecular (hydrophobic) interactions in which the rhamnose moieties might be involved (Table 2). Obviously, the polyelectrolyte effect dominates at low concentrations (very high dilutions), whereas the specific interactions prevail at higher concentrations.

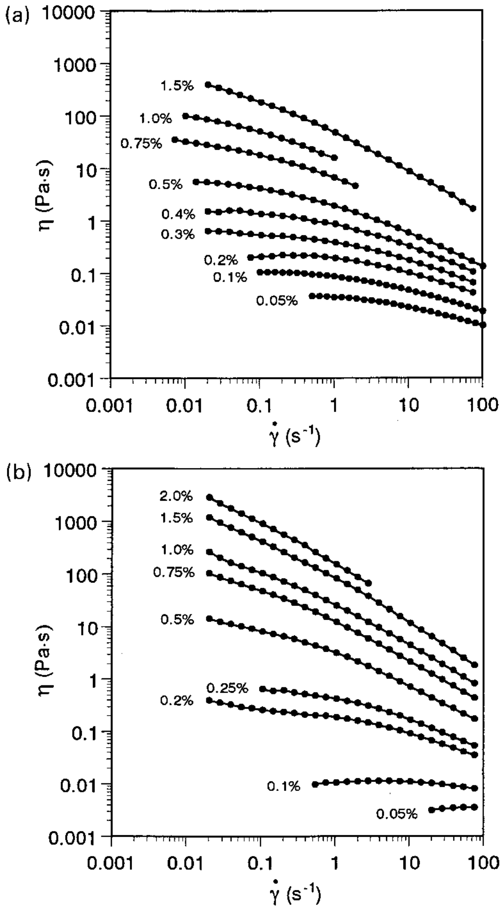

Fig. 3. The flow curves of 0.05, 0.1, 0.2, 0.25, 0.3, 0.4, 0.5, 0.75, 1.0, and 1.5% (w/v) of viilian in water (a). The flow curves of 0.05, 0.1, 0.2, 0.25, 0.4, 0.5, 0.75, 1.0, 1.5, and 2.0% in 0.1 M NaCl solution (b).

The complex dynamic viscosity (η^*) for the 1% viilian solution shows a power law dependence at frequencies higher than 1 rad/s (Fig. 2). Although the trace for the complex viscosity levels off at lower frequencies no real Newtonian plateau is reached within the range accessible by the rheometer. Also for the other concentrations a similar behavior of η^* is observed, however, the slope of these curves decreases with decreasing concentrations.

When we compare the characteristics of viilian with results obtained for other polysaccharides it appears that viilian complies with most criteria for a solution, however, the detailed shape of the mechanical spectra and the effect of NaCl suggest that viilian solutions cannot be described as true polymer solutions. The invariance of the limiting slopes of G' and G'' versus $\omega$with polysaccharide concentration in salt free solutions might be explained by the counteracting effects of intramolecular and intermolecular electrostatic repulsions due to the presence of phosphate groups, as was discussed earlier. Moreover, the effect of salt on the cross-over frequency of G' and G'' at higher polysaccharide concentration suggests that specific interactions are operative. However, aqueous solutions of viilian certainly do not obey the 'criteria' for a weak gel system (e.g., xanthan; Richardson & Ross-Murphy, 1987b). From the results presented here, it appears that viilian shows most resemblance to the exocellular polysaccharide of *Cyanospira capsulata* (Navarini, Cesàro & Ross-Murphy, 1992), which has a typical behavior intermediate between a liquid system (e.g., guar) and a weak gel. The special behavior probably results from a moderate chain flexibility together with weak interchain interactions. This assumption is in accordance with the primary structure of viilian (Nakajima et al., 1992). This structure (Fig. 1) combines a rather stiff backbone (Glc-β(1 → 4)-Gal-β(1 → 4)-Glc) with side chains of galactose–phosphate and rhamnose. Both moieties can facilitate a weak interaction which may be moderated by salt, either on the basis of ionic interactions (PO_4^-) or 'hydrophobic' interactions (rhamnose). The

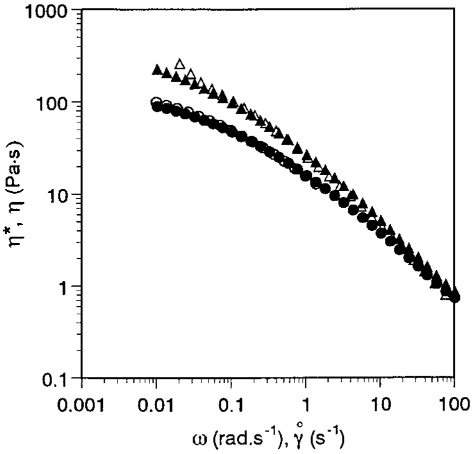

Fig. 4. The combined plot of dynamic viscosity (● ▲) and apparent viscosity (○ △) plotted against shear rate/radial frequency ('Cox–Merz plot') for 1.0% solutions of viilian in water (● ○) and in 0.1 M NaCl solution (▲ △).

importance of the phosphate groups is also illustrated by the observation that at low viilian concentrations, the addition of sodium ions results in a behavior that resembles a liquid system. Investigations of a dephosphorylated viilian will be conducted to obtain better insight in the role of the phosphate moieties. For similar reasons it is worthwhile to study the effects of salt type and concentration.

3.2. Steady shear experiments

Determination of the viscosity of a 1% aqueous viilian solution in steady shear flow clearly shows that the solution is shear-thinning or pseudoplastic (Fig. 3). At lower concentrations ($<$ 0.5%) the viscosity approaches to a Newtonian plateau when the shear rate is decreased (Fig. 3). However, for the 1% solution the Newtonian plateau is reached at shear rates well earlier 0.01 s^{-1}, which cannot be accessed as a result of the limited range of the apparatus. By increasing the shear rate, shear thinning (non-Newtonian) behavior of the viilian solution becomes apparent when the viscosity approaches a power law dependence with $\eta \propto \mathring{\gamma}^n$. At high shear rates, at a concentration of 1% (w/v), *n*, the slope of the flow curve (log η versus log $\mathring{\gamma}$ plot), has a value of $-$ 0.76, which is exactly the same as the slope found for commercial 'random coil' polysaccharides by Morris (1990). Moreover, this value is in agreement with the slopes found for flow curves of guar, xanthan, and the exocellular polysaccharide of *Cyanospira capsulata* determined in the same concentration regime, which are $-$ 0.79 (Robinson, Ross-Murphy & Morris, 1982), $-$ 0.80 (Milas, Rinaudo & Knipper, 1990), and $-$ 0.70 (Navarini et al., 1992), respectively. Also, these values compare perfectly with the slope predicted from the theory of Graessley for monodisperse polymers, which is $-$ 0.78 (Graessley, 1982).

In Fig. 4 it can be seen that the curve of complex viscosity versus radial frequency can be superimposed on the curve of steady shear viscosity versus shear rate. It thus appears that the solution obeys the so-called Cox–Merz rule (Cox & Merz, 1958), which indicates that no long range interactions are present that are disrupted in steady shear flow of the material.

Zero shear viscosities η_0 could be estimated from flow curves (Fig. 3) in the case of the lower concentrations. At higher concentrations (0.5% and up) for which a Newtonian plateau was not reached, it was possible to estimate a zero shear viscosity from a rate sweep experiment by extrapolation of the curves. Extrapolation of the determined data points to zero shear viscosity (Table 4) was accomplished by using the equation $\eta = \eta_0/[1 + (\mathring{\gamma}/\mathring{\gamma}_{1/2})^{0.76}]$, where $\mathring{\gamma}_{1/2}$ is the share rate at which viscosity is reduced to $\eta_0/2$, which was previously derived and applied by Morris (1990). Although there are several other such equations available, as Navarini et al. (1990) examined, the Morris equation appeared to be most suitable because there are less parameters involved and a reasonable fit with the flow curves could be obtained (Table 4). The zero shear viscosities determined are used to study the influence of the concentration of viilian on the properties of the solutions, both in water and salt. In Fig. 5 the plot of log η_0 versus the logarithm of the aqueous concentration is shown. Increasing the viilian concentration causes a discontinuity in the curve of

Table 4
The zero shear viscosities (η_0), shear rates giving $\eta_0/2(\mathring{\gamma}_{1/2})$ and the coefficients of correlation of Morris equations (Morris, 1990) fitted to the flow curves measured at different concentration of viilian solution

Sample (%)	Water solution η_0 (Pa s)	$\mathring{\gamma}_{1/2}$ (s^{-1})	r^2	0.1 M NaCl solution η_0 (Pa s)	$\mathring{\gamma}_{1/2}$ (s^{-1})	r^2
2	—	—	—	12 800	0.00354	0.999
1.5	673	0.0300	0.998	4 890	0.00439	0.999
1	122	0.0658	0.997	439	0.0240	0.990
0.75	37.7	0.101	0.990	172	0.0300	0.997
0.5	5.97	0.371	0.997	16.5	0.121	0.991
0.4	1.61	1.31	0.994	6.27	0.270	0.915
0.3	0.607	1.59	0.953	—	—	—
0.25	—	—	—	0.677	2.14	0.996
0.2	0.230	8.17	0.986	0.303	0.85	0.816
0.1	0.109	6.82	0.992	0.0111	246	0.674
0.05	0.039	16.4	0.991	0.0035	1 000	0.415

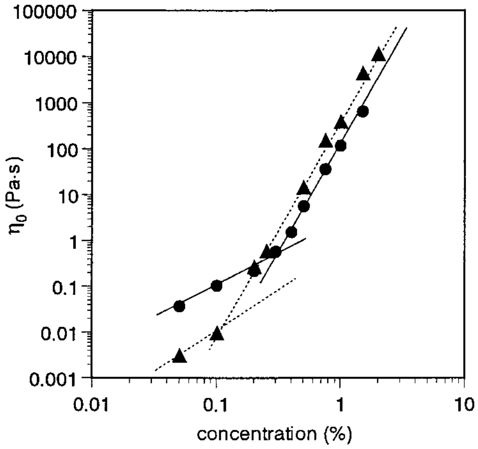

Fig. 5. The'zero shear' viscosity versus concentration of aqueous solutions of viilian (●) and solutions of viilian in 0.1 M NaCl (▲) in a double logarithmic plot.

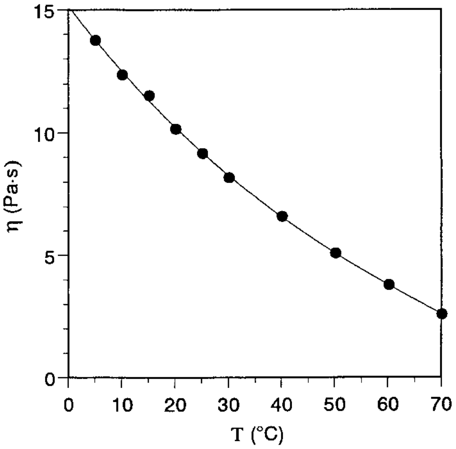

Fig. 7. The plot of steady shear viscosity (●) at a shear rate of 1 s^{-1} against temperature.

zero shear viscosity versus concentration. The concentration at which this sudden change in viscosity occurs, is the critical or total occupancy concentration (c^*) which relates to the volume taken by the polymer (Ross-Murphy, 1994). At concentrations earlier c^* the viscosity behavior is described by $\eta_0 \propto c^{1.17}$, which is in the same range $(1.1 > n > 1.4)$ as was observed for other polysaccharides (Milas et al., 1990; Ross-Murphy, 1994). At concentrations far beyond the critical concentration, the zero shear viscosity has a proportionality of approximately 4.5, which agrees well with the data from the literature (Castelain, Doublier & Lefebvre, 1987; Robinson et al., 1982). The presence of salt depresses the zero-shear viscosity in the dilute regime. This is almost certainly due to a suppression of the polyelectrolyte effect caused by the phosphate groups. However, at higher concentrations, the presence of salt enhances the viscosity in much the same way as discussed for the dynamic moduli. This effect could be tentatively explained as hydrophobic interactions appeared to be strengthened by suppressed ionic interactions under the presence of salt.

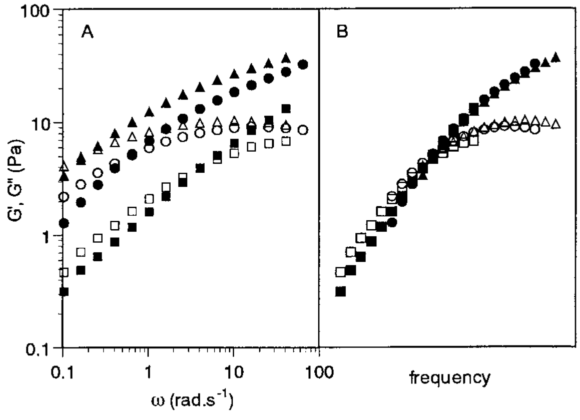

Fig. 6. (a) The frequency sweep for 1.0% aqueous solutions of viilian at 5°C (▲ △), 25°C (● ○), and 70°C (■ □). Storage modulus, G' (▲ ● ■); loss modulus, G'' (△ ○ □); (b) the G' and G'' curves are horizontally shifted to accomplish a master curve for each modulus.

3.3. *Effect of temperature*

An additional characteristic of an ideal polymer solution is that the viscoelastic spectra obtained at different temperatures can be superimposed by shifting the G' and G'' curves both horizontally and vertically (Ferry, 1980). Fig. 6 shows the mechanical spectra of 1% viilian in water at temperatures of 5, 25 and 70°C. Superimposition of both the G' and G'' curves accomplished by simply shifting horizontally seems to reasonably form a master curve for each (Fig. 6). Measurement of steady shear viscosity of the 1% viilian solution at different temperature in the range of 5–70°C shows a continuous relation but no drastic change in the curve (Fig. 7). These results suggest that no strong interaction or no conformational change took place between viilian molecules in the temperature range. From these observations, we can again conclude that viilian, unlike xanthan, can be regarded as a coiled polymer (Rochefort & Middleman, 1987) which does not show a temperature-dependent conformational change at least in the temperature range of 5–70°C.

4. Conclusions

Viscoelastic properties of the heteropolysaccharide viilian, which shows a remarkable effect on the consistency of fermented milk when produced in situ by *Lactococcus lactis* subspecies *cremoris* SBT 0495 (Toba et al., 1990), were studied in its partially purified form.

An aqueous solution of viilian behaves as an entangled polymer solution but not as a weak gel. The viscoelastic spectra of viilian solutions in the absence or presence of salt suggests that viilian shows the characteristics of a polyelectrolyte presumably due to the negative charge of the phosphate residue in the molecule. However, at higher concentrations, the lifetime of junctions is increased in the presence of salt which indicates that specific intermolecular interactions are involved. The dynamic viscosity and the steady shear viscosity superimposed in agreement with the Cox–Merz rule, indicating that in both flow patterns a similar type of molecular re-arrangement occurs. The dependence of viscosity on shear rate has a slope of approximately -0.8 at high shear rates which is in accordance with the behavior of random coil polymers. Moreover, viilian does not show a temperature-dependent conformational change in the temperature range of 5–70°C.

On comparison with the exocellular polysaccharide from *Cyanospira capsulata* it appears as if viilian can also be regarded as a random coil polysaccharide that, at higher concentrations, is able to form an entanglement network. This network is locally stabilized through specific non-covalent intermolecular interactions between semi-flexible segments of the polymeric chains, probably ionic (phosphate) and hydrophobic (rhamnose) interactions, both of which are influenced by the addition of salt.

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