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Influence of the drying method on the physical properties and immunomodulatory activity of the particulate (1 → 3)-β-D-glucan from *Saccharomyces cerevisiae*

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Abstract

The particulate (1 → 3)-β-D-glucan isolated from *Saccharomyces cerevisiae* cell walls was recovered from the aqueous medium as water-insoluble particles by different drying methods: solvent exchange (GE), lyophilisation (GL), and spray drying (GS). The samples were characterised by optical microscopy, FT-IR spectroscopy, swelling capacity, and rheological behaviour of aqueous dispersions. The immunological activity of the glucan samples was examined using the assay for the mitogenic and comitogenic activities. The drying method affected the microstructure of the glucan particles leading to differences in their physical properties (particle size and shape, swelling capacity, interparticle hydrogen bonding) as well as in the flow and viscoelastic properties. In comparison to GL and GE, the GS particles preserved the ellipsoid shape of yeast cells and exhibited a very low extent of interparticle hydrogen bonds. The rather liquid-like GS dispersion showed a several times lower apparent viscosity than the gel-like GE and GL dispersions. The results suggest that the physical state of the variously dried particulate glucan samples influenced significantly also their immunomodulatory activity which was found to be about twice higher with GS than with the GL and GE samples. The results indicated that for application of the particulate (1 → 3)-β-D-glucan as immunomodulator/adjuvans in form of aqueous suspension it is important to use spray-dried preparations.

Keywords: Particulate (1 → 3)-β-D-glucan; *Saccharomyces cerevisiae*; Drying effect; Hydrogen bonding; Rheology; Immunomodulatory activity

1. Introduction

In the last years, increased attention has been paid to various types of immunomodulating polysaccharides isolated from the cell walls of fungi and yeasts. The baker's yeast cell wall preparation—Zymosan was the first defined pharmaceutical yeast product with immunostimulatory activity (Fitzpatrick & DiCarlo, 1964; Pillemer & Ecker, 1941). Its active component was identified to be a β-D-glucan (Manners, Masson, & Patterson, 1973; Riggi & Di Luzio, 1961). Later, this water-insoluble polysaccharide, known as 'particulate glucan', was reported to posses phagocytic and candidacidal activities (Ferenčík et al., 1986), cytotoxic activity on mice peritoneal macrophages, and other biological activities (Bohn & BeMiller, 1995; Šandula, Machová, & Hříbalová, 1995; Tsiapali et al., 2001).

The fungal and yeast glucans have a common structure that comprises main chains of (1 → 3)-linked β-D-glucopyranosyl units along which are randomly dispersed side chains of β-D-glucopyranosyl units attached by (1 → 6) linkages (Kopecká, Phaff, & Fleet, 1974; Manners et al., 1973). Water solubility of the glucan depends on the frequency and length of the side chains. In contrast to the water-soluble glucans isolated from *Basidiomyces*, the particulate glucan from *Saccharomyces cerevisiae* is sparcely branched, i.e. contains single β-D-glucopyranosyl units on every eighth main chain unit (Kishida, Sone, & Misaki, 1992; Kogan, Alföldi, & Masler, 1988; Šandula, Kogan, Kačuráková, & Machová, 1999), rendering the glucan insoluble in water. β-Glucans are high molecular weight polymers with highly ordered helical structures, existing as single polymer strands with a helical conformation (single helix) or as a stable complex of three

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polymer strands forming a triple helix (Bohn & BeMiller, 1995).

Much controversy surrounds the biochemical and molecular principles of the immunostimulatory activity of β-glucans. According to some reports (Kishida et al., 1992; Ohno, Asada, Adachi, & Yadomae, 1995), a high molecular weight, a triple helix, and the distribution pattern of β-(1 → 6)-branches are the essential structural parameters, whereas other authors (Gomaa, Kraus, Franz, & Röper, 1991; Saito, Ohki, Takasuka, & Sasaki, 1977) suggest single helices. Demleitner, Kraus, and Franz (1992) reported the β-(1 → 3)-glycosidic linkage type to be the most important requirement for the activity. Contrary to the cited literature (Kishida et al., 1992; Kojima, Tabata, Ito, & Yanaki, 1986), the authors of a recent work (Kulicke, Lettau, & Thielking, 1997) came to the conclusion that helical structures are neither essential nor even advantageous for the immunological activity of β-glucans.

In any case, the expression of the immunological activity presupposes certain interactions between the polysaccharide and the macrophages or other cells of the biological systems studied. These interactions are dependent on the structural and molecular parameters of soluble β-glucans mentioned earlier. In the case of the water-insoluble β-glucan, such interactions would also be affected by the microstructure and the behaviour of the particles in aqueous dispersion used for applications. Our preliminary studies on the flow behaviour of the immunologically active, crude cell wall polysaccharides of *S. cerevisiae* have indicated differences related to the physical form of the samples (frozen, lyophilised, and sterilised). The aim of the present work was to investigate the influence of various methods of drying (solvent exchange, spraying and lyophilisation) on the physical structure and rheological properties of the β-glucan particles prepared from *S. cerevisiae* and find out whether their immunomodulating properties had been affected by the drying process.

2. Material and methods

2.1. Isolation of the (1 → 3)-β-D-glucan and preparation of differently dried samples

Commercial baker's yeast (*S. cerevisiae*) was purchased from Slovlik Trenčín (Slovakia). The water-insoluble (1 → 3)-β-D-glucan (particulate glucan) was obtained from the yeast by extraction with 6% NaOH at 60 °C for 4 h. Distilled water was added to the dispersion and the insoluble part after stirring for 30 min was collected by centrifugation. The sediment was suspended in 3% NaOH and heated at 90 °C for 2 h. The insoluble material was recovered by centrifugation, washed three times with distilled water and subsequently extracted twice with 4% phosphoric acid at room temperature for 2 h. More details on the extraction method had been described in a previous paper (Machová, Kogan, Alföldi, Šoltes, & Šandula, 1995). The insoluble residue, representing the cell wall (1 → 3)-β-D-glucan was separated by centrifugation, resuspended in distilled water and decanted with water until neutral reaction. Aliquots of the aqueous suspension were taken for the recovery of the particulate glucan using three different drying methods: lyophilisation (sample GL), precipitation with 4 volumes of 95% ethanol followed by washing of the precipitate three times with 95% ethanol and subsequent drying on air (sample GE), and the spraying technique (sample GS). The samples were stored in stoppered glass flasks and their residual moisture content of was determined after oven-drying at 100 °C to constant weight. The values found were 13.5, 12.7 and 11.0% for GS, GL, and GE, respectively.

2.2. FT-IR analysis

FT-IR spectra were collected on a NICOLET Magna 750 spectrometer with DGTS detector as an average of 128 scans and at a resolution of 4 cm^{-1}. The samples were dispersed in KBr (2 mg sample/200 mg KBr), ground, and pressed into pellets. Fourier self-deconvolution was applied using the OMNIC 3.2 software.

2.3. Characterisation of the microstructure

For optical microscopy measurements, the glucan sample was placed on agar layer and observed under a conventional light microscope with the enlargement of 16 × 100. The images were recorded by means of a COLOR VIDEO CAMERA-SONY connected to a computer.

2.4. Swelling capacity

The water uptake was determined after dispersing and swelling the glucan sample in distilled water (100 mg/14 ml) for 48 h at ambient temperature and occasional stirring. The volume occupied by the swollen particles was determined by weighing the sediment and the supernatant separated by centrifugation at 700 g for 15 min. The supernatant was removed by careful suction.

2.5. Rheological measurements

For the rheological testing, aqueous dispersions of the glucan samples were prepared at ambient temperature. The samples were preswollen in distilled water for 2 h and then stirred for 30 min. Then the dispersions were left to rest for 24 h at 10 °C before performing the measurements.

The flow properties of the dispersions were determined using the coaxial cylinder viscometer Rheotest 2 (2–50 Hz, VEB MLW Prüfgeräte Medingen, Germany) with the H measuring system ($r/R = 0.81$, $D = 0.17–146\ s^{-1}$). The shear rates were changed stepwise and applied until a constant shear stress value was obtained. The shearing time

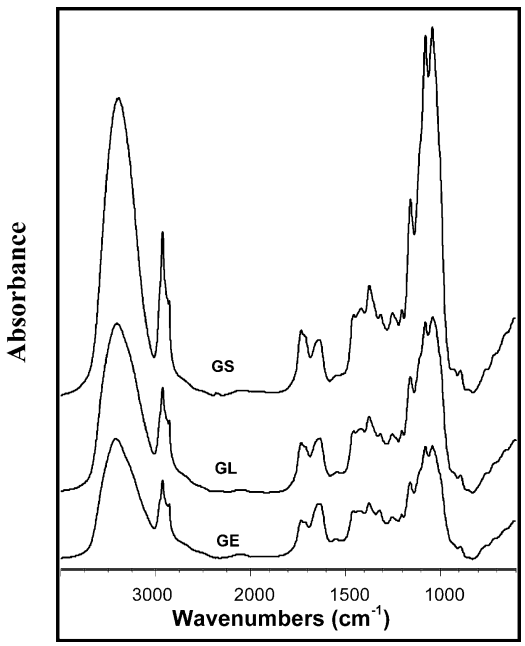

Fig. 1. FT-IR spectra (in KBr) of the particulate β-glucan samples dried by spraying (GS), lyophilisation (GL), and solvent exchange using ethanol (GE).

was about 15 min each for the ascending and descending shear cycles. The descending shear data were used to analyse the flow curves. The flow behaviour was described by the Oswald model: $\tau = KD^n$, where τ is the shear stress, D the shear rate, n the flow index (index of pseudoplasticity), and K the consistency index. The existence of structuration of shear-thinning dispersions is described also by the Casson model: $(\sigma)^{0.5} = (\sigma_{0c})^{0.5} + (\eta_\infty D)^{0.5}$, where σ_{0c} is the yield stress and η_∞ the infinite shear vicosity.

The viscoelastic behaviour of the dispersions was tested on the Rheometrics (RECAP II) TC-2000 (2K/2K) Sys IV using a disk and plate system (25 mm in diameter) with a gap of 1 mm. The rheological tests were performed at 1 Hz. Frequency sweep tests were conducted at 3% strain in the frequency range 10^{-1}–10^2 rad s^{-1}. The samples were covered with low-viscosity mineral oil to prevent the loss of solvent. The experiments were performed at 30 °C.

2.6. Mitogenic and comitogenic activity tests

Samples were subjected to an assay for their mitogenic and comitogenic activities as described in detail in a previous paper (Ebringerová, Hromádková, & Hříbalová, 1995). Briefly, the rat (strain Wistar, males weighing 200 g) thymocytes in medium RPMI-1640 supplemented with 10% fetal calf serum, used at a concentration of 1.5×10^6 cells per 0.2 ml per well, were stimulated by test compounds at concentrations 1, 10, 100, or 1000 μg/ml either in absence (mitogenic activity) or in presence (comitogenic activity) of PHA added to a final concentration 25 μ/ml. After 72 h cultivation, DNA synthesis was measured by the method of

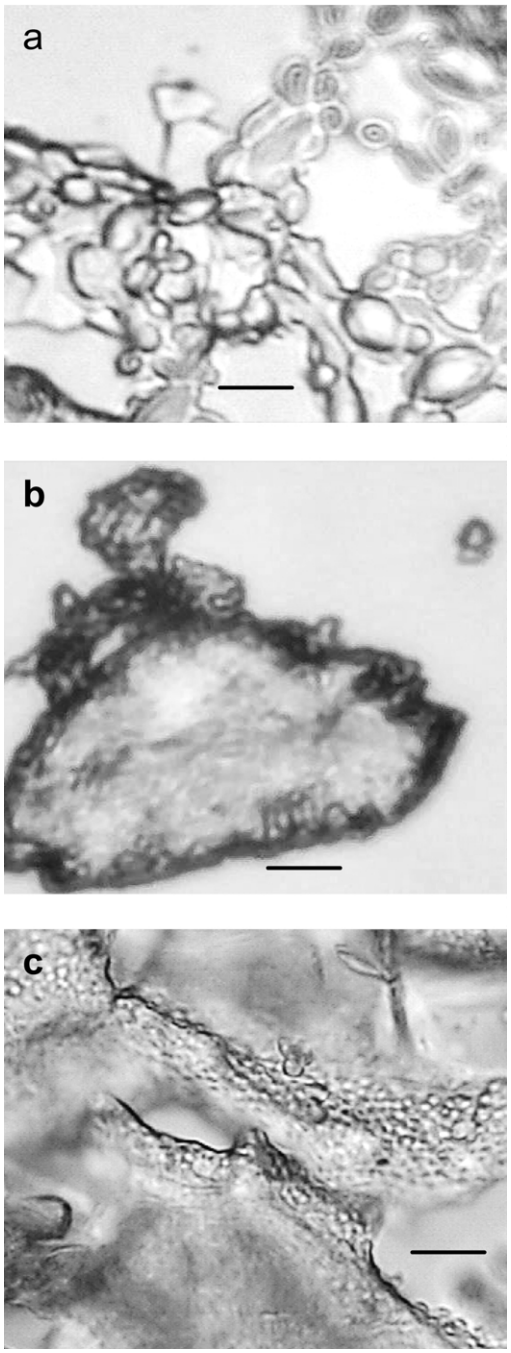

Fig. 2. Optical micrographs of particulate β-glucan samples (a) GS, (b) GE, and (c) GL. Bar = 10 μm.

^{3}H-thymidine incorporation. For calculation of the stimulation indices (SI), the means of the counts per min (cpm) for each four replicas were used. Possible contamination of the tested polysaccharides by endotoxin was checked by cultivation in the presence of polymyxin B (20 μg/ml) which inhibits, dose dependently, the biological effects of endotoxin, including its mitogenic activity (Jacobs & Morrison, 1977). The direct mitogenic effect of the compounds tested was expressed as: SI_{mit} = mean cpm for test compound/mean cpm without stimulant. The

comitogenic effect was expressed as: SI_{comit} = mean cpm (PHA + test compound)/mean cpm for PHA. In the repeated experiments, the mean cpm for control cultures was 912 ± 323 and that for cultures incubated with PHA was 1165 ± 622. The mean values of the samples were tested by the analysis of variance (ANOVA).

3. Results and discussion

3.1. Structural characterisation

The water-insoluble (1 → 3)-β-D-glucan was isolated from the yeast biomass by consecutive alkaline/acid extraction and purification steps in the form of an aqueous suspension of swollen particles. Different methods of drying, i.e. lyophilisation (GL), solvent exchange using ethanol (GE), and spray-drying (GS) should yield particles with unchanged chemical composition and structure of the glucan. In accord, the FT-IR spectra of the glucan samples (Fig. 1) showed the same spectral pattern typical of a (1 → 3)-β-D-glucan (Šandula et al., 1999), i.e. contained absorption bands arising from the ν (CC) and the ν (COC) stretching vibrations at 1160 cm^{-1}, two partially overlapped bands at 1078 and 1048 cm^{-1} attributable to ring and (C–OH) side group stretching, and a band at 891 cm^{-1} assigned to the β-glycosidic (C_1–H) deformation mode. The presence of amide I and amide II bands at 1655 and 1543 cm^{-1} accords with the residual protein content (1.6%) of the glucan. Because of the same protein content, the spectra of the three glucan samples were adjusted to the height of the amide bands. As seen in Fig. 1, the intensity of the other spectral bands ranged as follows: GE < GL ≪ GS. However, this might be affected by the different particle size and size distribution of the samples. Therefore, the glucan samples were further examined by optical microscopy.

As illustrated in Fig. 2, the particles obtained by the spraying process (GS) are elliptical with a rather compact, smooth surface and keeping the shape of the yeast cells. The particle size ranged between 2 and 10 μm. Probably, the original microstructure of the glucan particles was preserved due to the extremely fast evaporation of water molecules during the spraying process. Removal of water from the gel layer of the glucan particles by solvent exchange led to shrinking and deformation of the original particle surface combined with formation of particle aggregates. These newly formed particles (GE) showed about ten times larger sizes and had broader size distribution than observed with GS. In the case of lyophilisation, the water is freezed and removed as solid by sublimation. During this process, the surface of the GL particles is distored and compressed into sheet layers. Thus, the GL particles showed larger sizes and a porous surface. As seen, the different process of water removal had significantly affected the microstructure of the GL and GE particles in comparison to the GS ones. Thus, the effect in the case of the spray-drying process was very weak.

Polysaccharides are susceptible to hydrogen bond formation during drying from aqueous medium. Such physical changes can be studied by IR spectroscopy which has been applied in characterisation of hydrogen bonding and interactions in cellulose and modified cellulose (Cannon, 1959; O'Connor, 1972; Turhan, Sahba, & Güner, 2001) and other polymers (Cangelosi & Shaw, 1983). The most sensitive is the hydroxyl stretching region. Whereas the free hydroxyl groups absorb strongly in the 3650–3500 cm^{-1} region, hydrogen-bound hydroxyl groups cause shiftening of the band maximum position to lower frequencies, increase of intensity and/or broadening of the band as well as its symmetry distortion. Fig. 1 reveals significant differences between the three glucan samples in this spectral region. The FT-IR spectra of GE and GL showed broad ν (OH) bands with maxima at 3435 and 3415 cm^{-1} and a shoulder at 3250 and 3230 cm^{-1}, respectively, determined by Fourier self-deconvolution. The FT-IR spectrum of GS showed the highest intensity of the ν (OH) band and its maximum position shifted to lower frequency (3385 cm^{-1}). This can be explained by the smallest particle size of GS and the known highly ordered helical structure of the (1 → 3)-β-D-glucan molecules (Bohn & BeMiller, 1995) stabilised by a system of intra- and intermolecular hydrogen bonds. Vinogradov and Linnel (1971) used the parameter $\Delta_{1/2}$ to measure the ν (OH) band distortion in cellulose and modified celluloses. It was calculated from the differences between the distances measured from the vertical, constructed at band maximum, to the right and left within the range of 30–70% transmittance. The $\Delta_{1/2}$ values obtained for GE and GL, respectively, were 1.2 and 1.4 times higher than that of GS. The observed broadening and asymmetry of the ν (OH) band in the spectra of GE and GL suggests creation of new hydrogen bonds by strong interactions between particles in course of the corresponding drying processes what is in accord with the observed differences in the microstructure of the glucan particles (Fig. 2).

3.2. Swelling capacity and rheological properties

Water-insoluble polysaccharides can absorb and retain water, resulting in restricted swelling. The water uptake of the variously dried glucan samples was determined gravimetrically after redispersion of the particles in distilled water at constant conditions, followed by separation of the swollen particles by centrifugation. The water uptake of GL (12.4 g H_2O/100 g sample) was higher than those of both GE and GS (9.4 and 8.1 g/100 g, respectively). This might be related to the previously shown porous microstructure of GL particles able to retain water. It is to be mentioned that both GL and GE samples gave stable gel-like sediments after centrifugation, whereas the sedimented GS particles

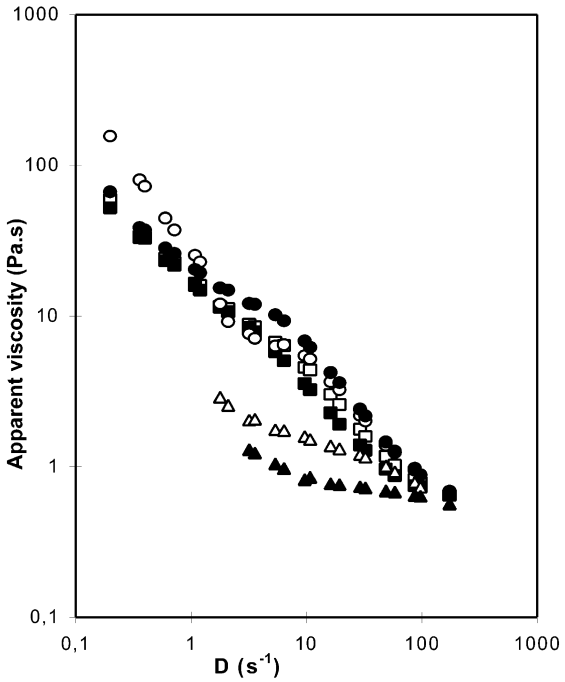

Fig. 3. Viscosity–shear rate of 13% (w/v) aqueous dispersions of GL (□), GS (△), and GE (○) measured at 20 °C. Empty symbols represent the ascending part and full symbols the descending part of the flow curves.

showed a tendency to redisperse very fast into the supernatant.

It can be expected that the observed interactions of the glucan particles with water molecules are reflected also in their rheological properties. As shown in Fig. 3, the aqueous dispersions of the variously dried glucan samples at the same polymer concentration ($C_p = 13\%$, w/v) showed a

Table 1
The Oswald and Casson parameters describing the shear stress versus shear rate data of the variously dried β-glucan samples at different temperatures

Glucan sample	T (°C)	Oswald model[a]			Casson model[b]		
		K (Pa s^n)	n	R^2	σ_{0c} (Pa)	η_∞ (Pa s)	R^2
GS	20	1.69	0.0810	0.99	0.056	0.618	1.00
	30	1.18	0.0833	1.00	0.042	0.467	1.00
	40	1.91	0.0747	1.00	0.096	0.475	1.00
	50	3.43	0.0626	0.99	2.255	0.444	1.00
GL	20	18.30	0.0287	0.98	14.741	0.282	0.91
	30	11.73	0.0323	0.98	8.612	0.275	0.96
	40	6.92	0.0415	0.98	4.230	0.321	0.99
	50	4.16	0.0529	0.99	2.799	0.348	1.00
GE	20	20.36	0.0306	0.97	16.705	0.339	0.87
	30	22.34	0.0361	0.94	19.192	0.482	0.76
	40	28.64	0.0345	0.94	25.184	0.529	0.74
	50	36.78	0.0301	0.93	32.780	0.486	0.72

The Oswald and Casson parameters calculated in the shear rate range 2–81 s^{-1}.

[a] $\tau = KD^n$.

[b] $(\sigma)^{0.5} = (\sigma_{0c})^{0.5} + (\sigma_\infty D)^{0.5}$.

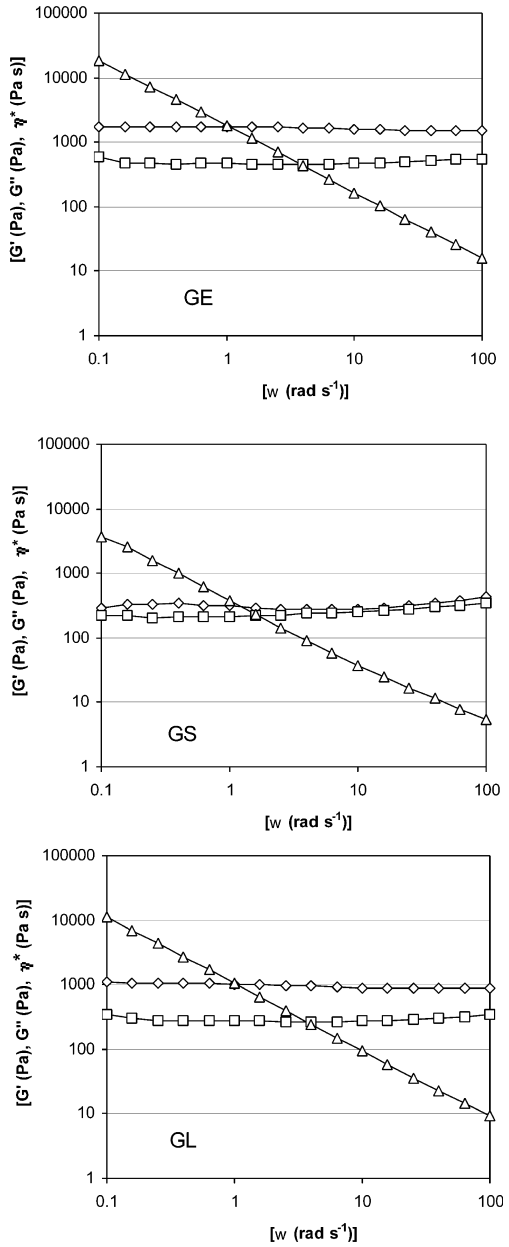

Fig. 4. Mechanical spectra of 13% (w/v) aqueous dispersions of β-glucan samples GS, GE, and GL at 30 °C. (△) Dynamic viscosity, η^*; (◇) storage modulus, G'; (□) loss modulus, G''.

non-Newtonian shear-thinning behaviour. In contrast to GS, the initial apparent viscosities (η_{app}) of the GE and GL dispersions were significantly higher and measurable at shear rates $D < 1\ s^{-1}$. With increasing D, the viscosity of GS reached a plateau at $D \sim 10\ s^{-1}$ and the corresponding value of η_{app} was very close to values attained by GE and GL at much higher shear rates ($D \sim 100\ s^{-1}$). The viscosity decrease can be ascribed to the existence of structuration in the dispersions (interparticle network formation) what is supported also by hysteresis of the flow curves (Fig. 3). The network is destroyed by shearing and only partially restored when the applied shear is removed. A particular behaviour was observed in the case of GE where the particles were

Table 2
Effect of the drying method on the mitogenic and comitogenic activities of the particulate β-glucan samples

Glucan sample	Dose (μg ml^{-1})					
	SI_{mit}			SI_{comit}		
	10	100	1000	10	100	1000
GS	2.54	8.43	18.37	6.71	37.55	64.94
GE	0.81	1.56	6.30	1.02	2.90	28.56
GL	1.11	2.32	5.96	0.95	3.00	32.97

In the repeated experiments, the mean cpm ± S.D. for control was 912 ± 323. For cultures incubated with PHA, the mean cpm ± S.D. was 1165 ± 622.

able to create a novel network in the medium range of the decreasing shear rate scan, resulting in a butterfly loop of the flow curve.

The flow behaviour of the glucan dispersions was measured in the temperature range 20–50 °C and was described by both the Oswald and Casson models. The calculated constants are summarised in Table 1. As seen, the Oswald function fits better the GE dispersion, giving higher regression values (R^2). The dispersions of all glucans tested showed pseudoplasticity expressed by the low values of the flow index (n) and yield stress (σ_{0c}). The σ_{0c} values of GL and GE exceeded that of GS by two orders, thus confirming the higher resistance of GL and GE dispersions against shearing. The glucan dispersions differed also significantly in the temperature-dependence of the flow behaviour. With increasing temperature, the consistency 'K' (corresponding to viscosity) of the GS dispersion decreased to a minimum at 30 °C and than rose up so that the value obtained at 50 °C was higher than that observed at 20 °C. In the case of GE, K showed a permanent increasing tendency with increasing temperature, whereas with the GL dispersion a decreasing tendency was observed. The yield stress of the dispersions followed the same temperature-dependences as their consistency values.

The unusual behaviour of GE resembles that of starch particles in aqueous dispersion (Sajjan & Rao, 1987) at elevated temperature. It can be explained by disaggregation of the glucan particles due to splitting of weaker interparticle hydrogen bonds by thermal treatment, resulting in enhancement of water uptake and viscosity increase.

Although all the tested glucan dispersions flow, their mechanical spectra obtained at 30 °C (Fig. 4) indicate gel-like properties: the storage modulus (G') was higher than the loss modulus (G'') over the accessible frequency range ($\omega = 0.1–100$ rad s^{-1}) and log η^* decreased linearly with increasing log ω, (Clark & Ross-Murphy, 1987). Although both moduli of all glucan dispersions are little frequency-dependent, the difference between G' and G'' are low and did not exceed one order in the case of GE and GL, what is indicative of a 'weak gel' behaviour. The viscoelasticity can be characterised by the phase angle ($\tan\delta = G''/G'$) (Cooney, Rosenberg, & Shoemaker, 1993). For typical polysaccharide gels $\tan\delta < 0.1$ (Morris, 1987). The phase angles of the GL and GE dispersions (0.35 and 0.30, respectively) indicate a gel-like behaviour. In contrast, $\tan\delta = 1.07$ found for GS indicates that the behaviour of this dispersion is closer to that of a liquid.

The GL and GE dispersions resemble that of the water-insoluble but swellable ispaghula husk flour which was reported (Haque, Morris, & Richardson, 1994) to behave as a weak gel. A common feature of polysaccharides with weak gel properties (Morris, 1987) is that they exist in solution as rigid ordered structures that form tenuous associations to give a continuous, but readily broken network. We assume that the rheological properties of the glucan samples are based, similarly as in the case of ispaghula husk flour, on network formation with structural rigidity at a supramolecular/interparticle level.

3.3. Immunological activity testing

The immunological activity of the glucan samples was characterised using the mitogenic and comitogenic in vitro thymocyte tests which were reported (Iribe & Koba, 1984) to show a positive correlation with the immunostimulatory effect in vivo in the case of muramyl glycopeptides and other substances. The use of polymyxin B in the culture medium did not indicate the presence of endotoxin in either of the samples tested. As shown in Table 2, the biological response of all polysaccharides in both the mitogenic and comitogenic tests was dose-dependent. The GS sample exhibited at doses 100 and 1000 μg/ml significantly higher response ($p < 0.05$) in both tests than those of the GE and GL samples showing similar immunomodulatory activities without significant differences ($p > 0.5$).

4. Conclusions

The results indicate that the drying process has a remarkable influence on the microstructure and properties of the glucan particles. The native state of the glucan particles was the best preserved during the spray drying. Removal of water by solvent exchange and lyophilisation enabled the formation of larger particle sizes and porosity of a sheet-like microstructure, respectively, connected with formation of interparticle hydrogen bond systems. These microstructural features affect significantly the rheological behaviour of the glucan particles in aqueous dispersion. The objective in making the comparison was to establish whether the method of drying might influence also the immunological activity. This appears to be the case and it was concluded that the activities of the variously dried glucan samples are closely related to their rheological behaviour. For preparing aqueous suspensions without the tendency to form associated structures and without the presence of larger sedimenting particles, the spray-dried

glucan sample is more suitable than the GE and GL samples. This may positively affect the interaction and/or diffusion processes of glucan particles in the biological testing systems. Two conclusions of practical importance need to be stressed:

for application of the particulate (1 → 3)-β-D-glucan in the form of aqueous suspension, the glucan preparation should be prepared by spraying drying,
for comparative biological studies of glucan preparations, it is necessary to use samples prepared by the same drying process.

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