Eur Biophys J (2008) 37:503–508
DOI 10.1007/s00249-007-0245-0

ORIGINAL PAPER

Protection of liposomes against fusion during drying by oligosaccharides is not predicted by the calorimetric glass transition temperatures of the dry sugars

Dirk K. Hincha · Petra Rennecke · Ann E. Oliver

Received: 7 September 2007 / Revised: 14 November 2007 / Accepted: 20 November 2007 / Published online: 7 December 2007

Abstract Sugars play an important role in the desiccation tolerance of most anhydrobiotic organisms. It has been shown in previous studies that different structural families of oligosaccharides have different efficacies to interact with phospholipid headgroups and protect membranes from solute leakage during drying. Here, we have compared three families of linear oligosaccharides (fructans (inulins), malto-oligosaccharides, manno-oligosaccharides) for their chain-length dependent protection of egg phosphatidylcholine liposomes against membrane fusion. We found increased protection with chain length up to a degree of polymerization (DP) of 5 for malto-oligosaccharides, and a decrease for inulins and manno-oligosaccharides. Differential scanning calorimetry measurements showed that for all sugars the glass transition temperature (T_g) increased with DP, although to different degrees for the different oligosaccharide families. Higher T_g values resulted in reduced membrane fusion only for malto-oligosaccharides below DP5. Contrary to expectation, for inulins, manno-oligosaccharides and malto-oligosaccharides of a DP above five, fusion increased with increasing T_g, indicating that other physical parameters are more important in determining the ability of different sugars to protect membranes against fusion during drying. Further research will be necessary to experimentally define such parameters.

D. K. Hincha
Max-Planck-Institut für Molekulare Pflanzenphysiologie,
Am Mühlenberg 1, 14476 Potsdam, Germany

P. Rennecke
School of Veterinary Medicine,
University of California, Davis, CA 95616, USA

A. E. Oliver
Department of Applied Sciences,
University of California, Davis, CA 95616, USA

D. K. Hincha (✉)
Max-Planck-Institut für Molekulare Pflanzenphysiologie,
Am Mühlenberg 1, 14424 Potsdam, Germany
e-mail: hincha@mpimp-golm.mpg.de

Abbreviations

DP	Degree of polymerization
EPC	Egg phosphatidylcholine
Fru	Fructose
Gal	Galactose
Glc	Glucose
Man	Mannose
NBD-PE	*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine
RH	Relative humidity
Rh-PE	*N*-(lissamine rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine
RFO	Raffinose-family oligosaccharides
Suc	Sucrose
T_c	Critical temperature
TEN	TES–EDTA–NaCl buffer
*T*g	Calorimetric glass transition temperature

Introduction

Sugars have the ability to stabilize biological cells during desiccation (see Crowe et al. 1992 for a review). In this regard, the effects of sugars on the stability of liposomes, which are a convenient model of biological membranes, during drying have been extensively investigated (see Hincha et al. 2006 for a recent review). Much of this research has focused on the disaccharides Suc and trehalose, while other potential protectants, such as oligosaccharides, have

received less attention (see Oliver et al. 2002, 2001 for reviews).

It has, however, been shown that manno-oligosaccharides (linear oligomers of β1-4 linked Man; Man–Man$_n$; with n = number of monosaccharide units) (Cacela and Hincha 2006b) and malto-oligosaccharides (linear oligomers of α1-4 linked Glc; Glc–Glc$_n$) (Cacela and Hincha 2006b; Hincha et al. 2002; Suzuki et al. 1996) decrease in their effectiveness to protect liposomes from leakage of a soluble marker during drying with increasing DP. RFO (linear oligomers of Gal α1-6 linked with a capping Suc; Glc–Fru–Gal$_n$) (Hincha et al. 2003) and inulins (linear oligomers of Fru β2-1 linked with a capping Glc; Glc–Fru$_n$) (Cacela and Hincha 2006b; Hincha et al. 2002), on the other hand, become more effective with increasing DP. Recently, it has been shown that the branched fructans from cereals such as oat and rye show a more complex dependence of membrane stabilization on DP, with optima at intermediate chain lengths (Hincha et al. 2007).

The differences between the different linear oligosaccharides have been related to their ability to interact with the lipid headgroups in the dry state. In particular, differences in the extent of H-bonding of different oligo-saccharides of the same DP to the P = O groups (Cacela and Hincha 2006a, b; Hincha et al. 2002, 2003) have been related to the differential protection of liposomes from leakage during drying. Similar differences have been reported for interactions with the more exposed choline group of EPC, but not for the more buried C = O group (Cacela and Hincha 2006b). Such H-bonding interactions of sugar OH-groups with the lipid headgroups are thought to replace hydration water of the lipids during drying (Oliver et al. 1998a) and are closely related to the ability of different sugars to depress the lipid liquid–crystalline to gel phase transition temperature in the dry state, similar to the effect of water (Cacela and Hincha 2006a).

Another important determinant of membrane stability during drying is the prevention of membrane fusion between liposomes, which can also lead to solute leakage. It is generally accepted that sugars prevent liposome fusion during drying by forming a glass (vitrification) and thereby preventing the close approach of vesicles necessary for fusion, without the need for specific interactions with the membranes (see Buitink and Leprince 2004; Crowe et al. 1998 for reviews). The ability of a substance to vitrify can be experimentally characterized by its T_g. In oligo- and polymers, T_g generally increases with molecular weight (Slade and Levine 1991) and an increase in T_g with DP has been reported for RFO (Buitink et al. 2000a), fructans (Hinrichs et al. 2001; Schaller-Povolny et al. 2000), and malto-oligosaccharides (Orford et al. 1989). However, while RFO and malto-oligosaccharides induced a decrease in membrane fusion with increasing DP (Hincha et al. 2002, 2003), fusion increased with DP in the presence of inulins (Hincha et al. 2002). These effects on fusion are notably different from the effects on leakage and the ability of the oligosaccharides to H-bond to lipid headgroups, indicating that different properties of the sugars may be responsible for their effects on leakage and fusion.

In the present paper we report a systematic study of the effect of DP on the ability of inulins, malto-oligosaccharides and manno-oligosaccharides to prevent liposome fusion during drying and compare these data with the T_g values of the respective sugars. Surprisingly, our analysis shows that while T_g increased with DP in all three oligosaccharide families, membrane fusion decreased only in the presence of malto-oligosaccharides up to DP5. In the presence of inulins, manno-oligosaccharides and higher DP malto-oligosaccharides fusion increased both as a function of DP and T_g.

Materials and methods

Materials

EPC was purchased from Lipid Products (South Nutfield, Surrey, UK). NBD and Rh-PE were obtained from Molecular Probes (Leiden, The Netherlands). Suc and malto-oligosaccharides were obtained from Sigma, inulins and manno-oligosaccharides were purchased from Megazyme (Wicklow, Ireland). The purity of the sugars was at least 95%, as determined by analytical HPLC, as described in detail in previous publications (Hincha et al. 2002, 2003).

Preparation of liposomes

Lipids were dried from chloroform under a stream of N_2 and stored under vacuum overnight to remove traces of solvent. Liposomes were prepared from lipids hydrated in TEN buffer (10 mM TES, 0.1 mM EDTA (pH 7.4), 50 mM NaCl), using a hand-held extruder with two layers of polycarbonate membranes with 100 nm pores (MacDonald et al. 1991; Avestin, Ottawa, Canada).

Membrane fusion experiments

Liposome fusion after drying and rehydration was determined using fluorescence resonance energy transfer (Struck et al. 1981) as described in detail before (Hincha et al. 1998). Briefly, two EPC liposome samples were prepared: one sample was labeled with 1 mol% each of the fluorescent probe pair NBD-PE and Rh-PE, while the other sample was unlabeled. The two samples were combined after extrusion in TEN buffer in a 1:9 (labeled:unlabeled) ratio, resulting in a final lipid concentration of 10 mg/ml.

Liposomes (40 µl) were mixed with an equal volume of concentrated solutions of sugars in TEN and 20 µl aliquots were filled into the caps of microcentrifuge tubes. The samples were dried in desiccators at 28°C and >1% RH for 24 h in the dark (Hincha et al. 2002). Fusion was measured after rehydration in TEN buffer by fluorescence resonance energy transfer (Struck et al. 1981) with a Kontron SFM 25 fluorometer (Kontron Instruments, Neufahrn, Germany) at excitation and emission wavelengths of 450 and 530 nm, respectively. Due to a slight quenching effect of Triton-X 100 on NBD fluorescence, fusion values above 100% are calculated (Fig. 1) for fully fused liposomes (see Tomczak et al. 2001 for details).

Differential scanning calorimetry of sugar glasses

Glass transitions (T_g) were measured using a Perkin Elmer DSC-7 (Norwalk, CT, USA) or a Mettler–Toledo DSC822 differential scanning calorimeter (Columbus, OH, USA), both equipped for subambient operation. Sugar solutions were dissolved in 18 MΩ water at 200–500 mg/ml and air dried into Differential scanning calorimetry (DSC) pans in a sealed box that was continuously purged with dry air of less than 3% RH over a period of 2–3 days. Finally, samples were incubated for ~1 h at elevated temperature (60–80°C) to remove residual water and pressure-sealed in the dry box. For the state diagram, samples were transferred to chambers maintained at specific RH over saturated salt solutions (Weast 1984) for 1–24 h before sealing. Samples were scanned in the range of −50 to 200°C at 10°C/min. Glass transitions were analyzed using the Perkin Elmer or Mettler software, which determine T_g as the stable midpoint of the baseline inflection. Every experiment was performed twice, with at least three heating runs for each experiment. Water contents were determined gravimetrically using a Cahn C-33 electrobalance, as previously described (Oliver et al. 1998b).

Results and discussion

We have investigated the effects of three structural families of oligosaccharides, inulins, malto-oligosaccharides, and manno-oligosaccharides, on membrane fusion in the dry state. While fusion data have been reported previously for the first two families (Hincha et al. 2002), the last has not

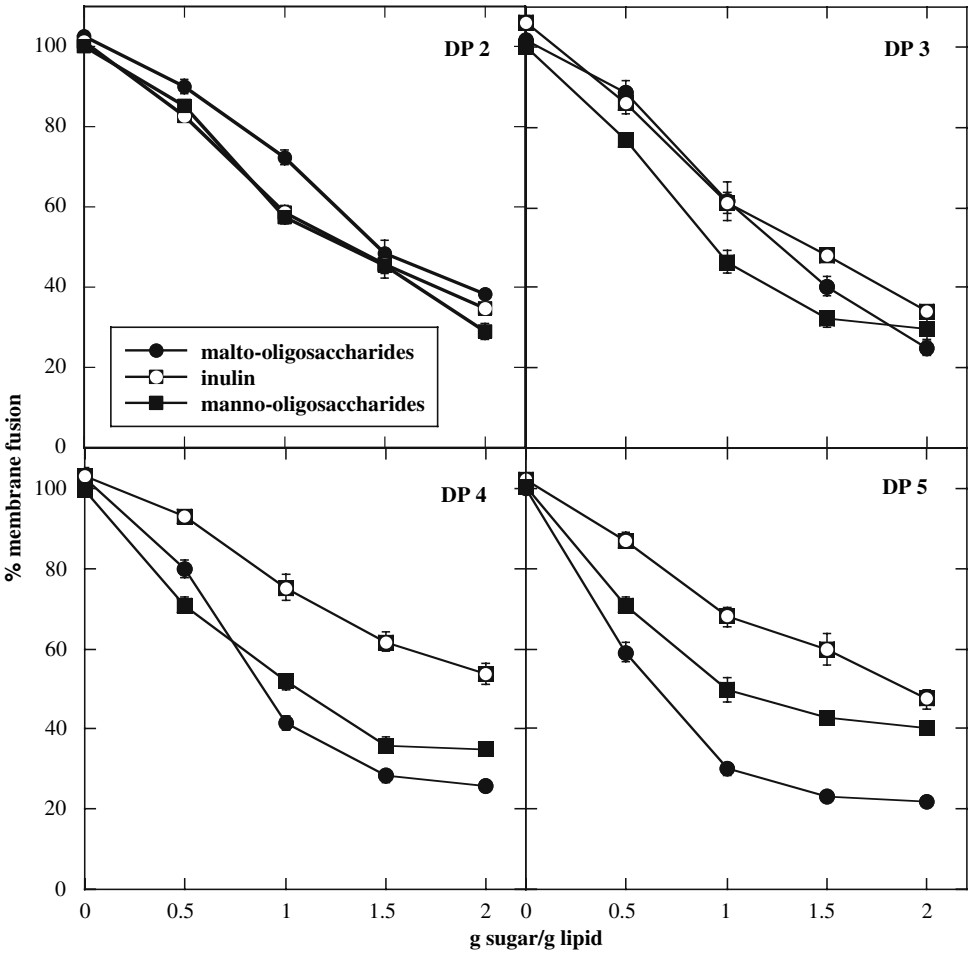

Fig. 1 Protection of large unilamellar liposomes from damage during drying by inulins, malto-oligosaccharides and manno-oligosaccharides of different degrees of polymerization (*DP*). Fusion of the vesicles after air-drying and rehydration is shown as a function of the concentration of the sugars in the suspending medium. Data points represent means ± SEM from three parallel samples. The data for inulins and malto-oligosaccharides were taken from Hincha et al. (2002)

been investigated previously. We used commercially available sugars from DP2 to DP5 for inulins and manno-oligosaccharides, and DP2 to DP7 for malto-oligosaccharides.

Figure 1 shows the effects of oligosaccharides up to DP5 on liposome fusion after drying and rehydration. All sugars showed concentration dependent protection of liposomes against fusion. The degree of protection, however, varied with DP and oligosaccharide family. While the malto-oligosaccharides showed increased protection up to DP5, the members of the other two families showed a linear decrease in protection with increasing DP (Figs. 1, 2). However, at higher DP (DP6 and 7), the effectiveness of the malto-oligosaccharides also decreased (Fig. 2). These experiments have identified, in addition to data reported before for inulins and malto-oligosaccharides (Hincha et al. 2002), manno-oligosaccharides as another family of sugars that shows less inhibition of fusion with increasing DP. The effect of DP was not as strong as for the inulins, but clearly in the opposite direction than for the malto-oligosaccharides up to DP5 (Figs. 1, 2).

The basic assumption about the role of sugars in preventing membrane fusion during drying is that it is due to the formation of a glass phase (see Crowe et al. 1998; Hincha et al. 2006 for reviews). In addition, it has been shown that heating samples containing Suc above the sugar glass transition temperature leads to a rapid increase in membrane damage (Sun et al. 1996). Therefore, we compared the vitrification behavior of the oligosaccharides by DSC to elucidate the interdependence of membrane fusion, DP, and T_g.

Figure 3 shows state diagrams for the glass transitions of manno-oligosaccharides, malto-oligosaccharides and inulins of DP5. In common with other sugar glasses (Buitink and Leprince 2004; Crowe et al. 1998), water acted as a plasticizer and led to a progressive decrease in T_g with increasing water content. The degree of reduction in T_g, however, was different for the different oligosaccharides, in accordance with findings for other oligo- and polysaccharides (Sun et al. 2004). It was not possible to obtain a T_g value for extremely dry (below 0.13 g water/g dry weight) DP5 manno-oligosaccharide, because the sample decomposed during heating before T_g was reached. However, the data suggest that at a given water content the DP5 inulin showed the lowest and the corresponding manno-oligosaccharide the highest T_g, with the malto-oligosaccharide intermediate.

For all three families of oligosaccharides, T_g increased with DP, as expected (Fig. 4a). The increase was highest for the malto-oligosaccharides and lowest for the inulins, with the manno-oligosaccharides intermediate. The caveat in the case of the manno-oligosaccharides is; however, that in the DP5 sample, water content was higher than in all other samples (compare also Fig. 3), so that T_g may be underestimated in this case. Similarly, T_g was slightly higher for the DP2 than for the DP3 manno-oligosaccharide, indicating that its water content was lower.

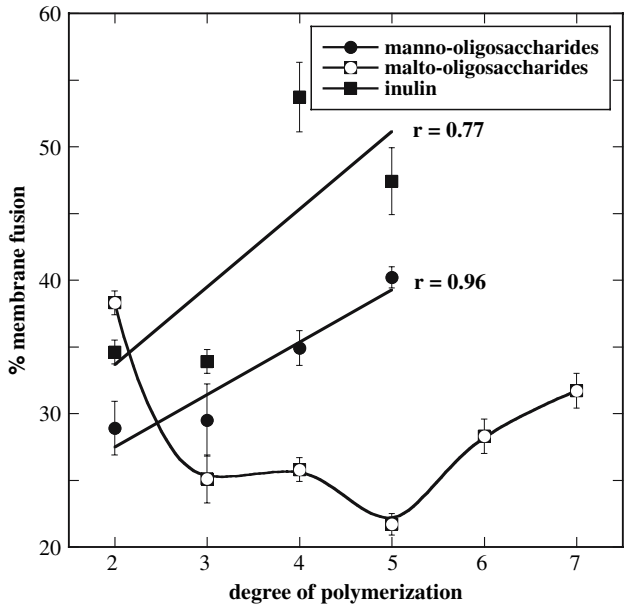

Fig. 2 Percentage of liposome fusion after air-drying and rehydration as a function of the DP of inulins, malto-oligosaccharides and manno-oligosaccharides. All samples contained 2 g sugar/g lipid (compare Fig. 1). The straight lines for samples containing inulins and manno-oligosaccharides were fitted by least-squares linear regression. The regression coefficients (r) are shown next to the respective lines. The line connecting the data from the malto-oligosaccharide containing samples is only meant as a visual aid. The data for inulins and malto-oligosaccharides were taken from Hincha et al. (2002)

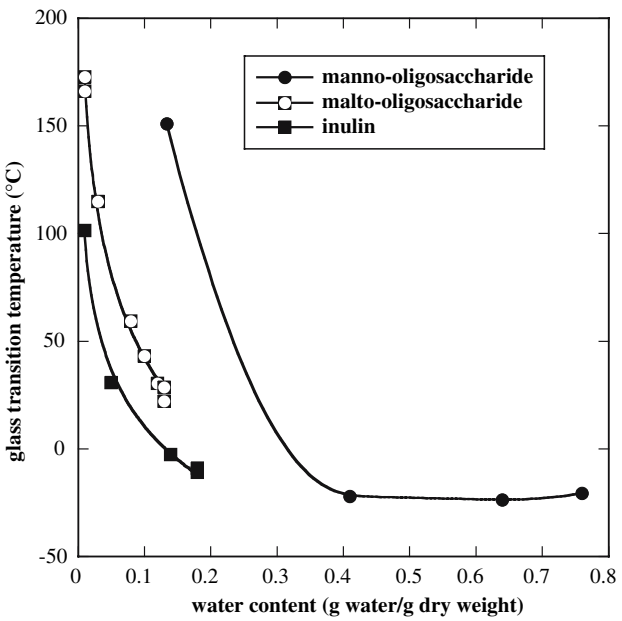

Fig. 3 State diagram of DP5 sugars of different oligosaccharide families as a function of water content in the samples

Unfortunately, the sugar in this sample decomposed during gravimetric analysis, so that no exact water content data are available. However, a sample of DP2 manno-oligosaccharide with a higher-water content (0.26 g water/g dry weight) showed the expected lower T_g (27.4°C). In addition, double reciprocal plots (Fig. 4b) yielded the expected straight lines for all oligosaccharide families (Orford et al. 1989), indicating that the data reflect the relative vitrification behavior of the sugars reasonably well.

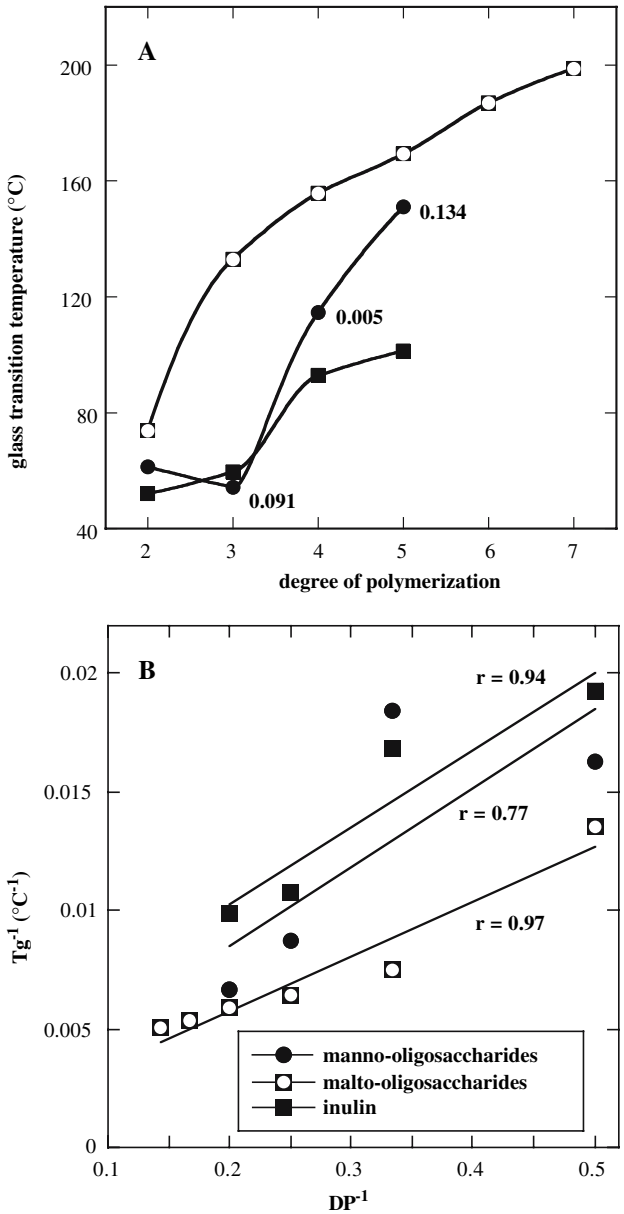

Fig. 4 Glass transition temperatures of sugars from the different oligosaccharide families as a function of DP(**a**). Panel (**b**) shows a double reciprocal plot of the data in **a**. The water content of the samples was below 0.01 g water/g dry weight for all inulins and malto-oligosaccharides, but is indicated in the figure for the manno-oligosaccharides of DP3–DP5 (see text for further details)

A plot of membrane fusion as a function of the T_g of the different sugars presents a surprising picture (Fig. 5). Only malto-oligosaccharides up to DP5 show the expected behavior, i.e. a reduction in fusion with increasing DP. The higher DP malto-oligosaccharides and all other oligosaccharides, on the contrary, show an increase in membrane fusion with increasing T_g. On the other hand, it has been shown before that sorbitol is a much better protectant against membrane fusion during drying than would be expected from its T_g (Hincha and Hagemann 2004). It has been suggested previously that T_c, the critical temperature where a glass changes from the solid-like to the liquid-like state, may be a better parameter for the functional characterization of sugar glasses (Buitink et al. 2000b). T_c for sorbitol, for example, is approximately 40°C above T_g (Tsujimi et al. 1999a, b), while it is only 18°C above T_g for sucrose (Buitink et al. 2000b). Unfortunately, no T_c data for the oligosaccharides investigated in our study have been published.

In addition, it has been shown in recent spectroscopic investigations that there is spatial and temporal heterogeneity in sugar glasses, even at temperatures considerably below T_g and that this heterogeneity is different for different sugars and sugar alcohols (Shirke and Ludescher 2005a, b).

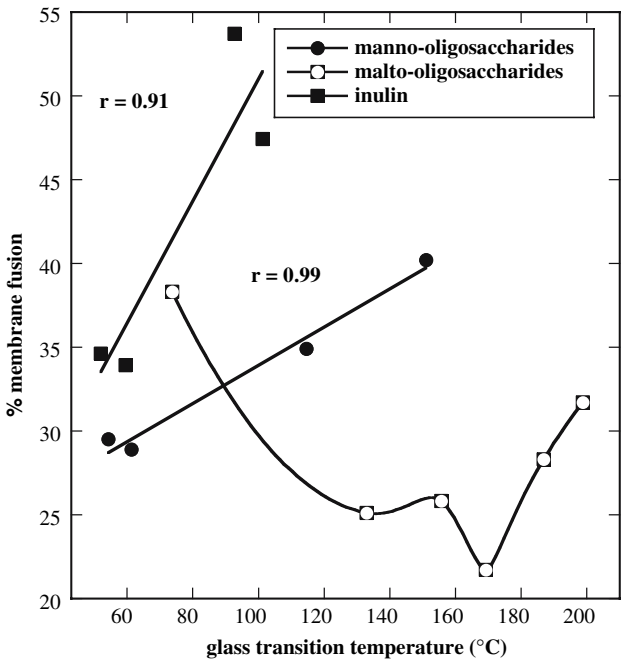

Fig. 5 Percentage of liposome fusion after air-drying and rehydration as a function of the glass transition temperature of the respective oligosaccharides of different DP. The fusion data are taken from samples that contained 2 g sugar/g lipid (compare Figs. 1, 2). The *straight lines* for samples containing inulins and manno-oligosaccharides were fitted by least-squares linear regression. The regression coefficients (r) are shown next to the respective lines. The line connecting the data from the malto-oligosaccharide containing samples is only meant as a visual aid

Conclusions

While there is little doubt that vitrification of the sugar matrix during drying is necessary to prevent membrane fusion (Buitink and Leprince 2004; Crowe et al. 1998) and that fusion is increased when samples are heated above T_g (Sun et al. 1996), the present study shows that measurements of T_g are not a good predictor of membrane protection against fusion for different sugars. This is even true for oligosaccharides of the same DP, so that effects of size differences can be largely excluded. Therefore, our data indicate that structural differences between different sugars play an important role not only in the ability of sugars to interact with lipid headgroups (Cacela and Hincha 2006b), but also in their ability to form a glass that effectively prevents membrane fusion. Further investigations will be necessary to relate structural differences between different sugars to functional differences, to understand the structural basis of the protective abilities of different oligosaccharides.

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