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Biochimica et Biophysica Acta 1375 (1998) 36–42

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Fructans interact strongly with model membranes

R.A. Demel [a,*], E. Dorrepaal [b], M.J.M. Ebskamp [b], J.C.M. Smeekens [b], B. de Kruijff [a]

[a] *Department Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, Netherlands*

[b] *Department of Molecular Cell Biology, Institute of Biomembranes, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, Netherlands*

Received 18 May 1998; revised 21 July 1998; accepted 4 August 1998

Abstract

Bacterial fructans with a high degree of polymerisation cause a very large increase in surface pressure of lipid monolayers at the air–water interface with a broad range of lipids, including phosphatidylethanolamine and several types of phosphatidylcholines. The surface active effect of fructans contrasts strongly with the maximal effects observed for trehalose, sucrose and glucose under comparable conditions (20 and 0.6 mN/m for fructans and the other sugars, respectively). The results demonstrate a profound and specific membrane interaction of the fructans which is probably very different from the effect of the smaller carbohydrates. The fructan concentrations used in this study are within the physiological range observed in fructan-accumulating plants. The suggested water-stress protective effect of fructans may be induced by membrane–fructan interaction which prevent lipid condensation and phase transitions to take place.

Keywords: Monomolecular layer; Carbohydrate; Fructan; Desiccation; Phase transition; Phospholipid

1. Introduction

Plant growth and productivity are limited more by water deficit than by any other environmental variable, including disease. During evolution, diverse strategies for the protection of plants against the adverse effects of drought have emerged. One such strategy involves the biosynthesis of molecules which protect vital structures in the plant cell against drought, especially membranes and proteins.

Fructans (fructose polymers) are one group of compounds for which a role as water stress protectant has been suggested for many years [1–3]. This suggestion is based on the distribution of fructan-accumulating plants in dry and cold environments and a steep increase in fructan content in these species in cold and dry conditions.

Recent studies on drought tolerance of transgenic tobacco plants [4] suggest that fructans can protect plants against water stress. These plants harbor microbial fructosyltransferase genes and accumulate high molecular weight (DP > 25 000), mostly β (2→6)-linked microbial type fructans. When exposed to drought, fructan-accumulating transgenics have a higher growth rate and biomass production in comparison with wild-type plants. The differences are greatest for the root system. At optimum water supply the development of transgenics and wild-type plants is identical. In these plants, fructan accumu-

* Corresponding author. Fax: +31 (30) 252-2478; E-mail: r.a.demel@chem.uu.nl

0005-2736/98/$ – see front matter
PII: S0005-2736(98)00138-2

lates at low levels and does not significantly contribute to the osmotic potential.

Loss of selective barrier function of biological membranes is an important cause for loss of viability due to drought or cold. We were therefore interested to test whether fructans can interact with and possibly protect membranes.

To study possible membrane interactions of fructans, monomolecular layers were used as a model membrane. Monomolecular layers at the air–water interface have proven to be of great value in the study of membrane-interacting compounds [5]. A special advantage of monolayers is the ability to vary the molecular packing of the lipid constituents. Changes in surface pressure indicate membrane insertion.

In our search for a molecular explanation for the drought-protective action of fructans, we discovered that fructans interact with lipid membranes in a strong and specific way. Such interactions could well protect cells and organisms against the harmful consequences of dehydration by insertion of fructan molecules into and interaction with the lipid head group region.

2. Materials and methods

The following types of fructans were used: fructans of the levan-type isolated from the medium of *Bacillus subtilis*; fructans of low molecular weight (DP 3–5) were obtained from Meioligo (Meiji Seika Kaisha Saitama Japan); fructans of the inulin-type isolated from *Helianthus tuberosus* and from dahlia tubers were obtained from Sigma (St. Louis, USA). These fructans were purified on HPLC and the molecular mass was analyzed. The degree of polymerization ranged between 3 and 35 (*H. tuberosus)* and between 10 and 35 fructose residues (dahlia); trehalose, sucrose, fructose and glucose were obtained from Sigma; dipalmitoylphosphatidylcholine (DPPC); dimyristoylphosphatidyl-ethanolamine (DMPE); and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids.

2.1. Isolation and purification of fructans from *Bacillus subtilis*

Fructans were isolated from the supernatant of a *B. subtilis* 1A165(QB13) culture. The culture was inoculated in 200 ml Spizizen salt medium with sucrose and tryptophan and incubated for 72 h at 30°C. The induction of sacB by sucrose led to the production of levansucrase followed by the synthesis of fructan in the medium. The suspension was centrifuged for 10 min at $4500\times g$, whereafter fructans were isolated from the supernatant by two different methods.

2.1.1. Method A

Ethanol was added to the supernatant to a final concentration of 90% v/v. The fructans were allowed to precipitate at 4°C for 4 h, isolated by centrifugation for 10 min at $9000\times g$ and dried in a vacuum desiccator. The fructans were dissolved in 10 ml milliQ-water and incubated with proteinase K (50 μg/ml proteinase K, 10 mM Tris pH 7.5, 5 mM EDTA) for 2 h at 55°C. The ethanol precipitation was repeated and the fructans were dissolved in milliQ-water.

2.1.2. Method B

From the supernatant of the *B. subtilis* culture first levansucrase was removed by the addition of ethanol to a concentration of 48% v/v. Fructans were isolated by increasing the ethanol concentration to 80% v/v. After overnight precipitation at 4°C and centrifugation for 10 min at $9000\times g$ the pellet was dried. Fructans were fractionated by FPLC on Sephacryl S200-HR and further checked by TLC with *H. tuberosus* fructans as a referent material. The TLC plates were run three times in 87% acetone–water and spotted with urea by heating to 80°C for 20 min.

The size of *H. tuberosus* and dahlia fructan was analyzed on HPLC, CarboPac PA-100, by comparison with a series of fructans from chicory.

During all growth and isolation procedures the use of surface active compounds was avoided regarding the surface pressure measurements.

2.2. Monolayer experiments

2.3. Measurements at constant surface area

The surface pressure which is defined as the difference of the surface-tension of water and the surface-tension of the film-covered interface was measured by the Wilhelmy method [5]. Experiments were performed at 21°C and lipid monolayers were formed

on a subphase of 5 ml. MilliQ-water to an initial surface pressure between 15–35 mN/m. Saccharides were added through a small injection hole to the subphase which was continuously stirred. The change in surface pressure after the addition of the saccharide to the subphase was taken as a measure for the interaction of the saccharide with the lipid phase. The surface pressure increase was measured in time until a stable surface pressure was reached. The surface activity that could be developed by the saccharide itself was measured in the absence of a lipid film.

2.4. Compression of monomolecular layers

Pressure–area curves were measured after spreading 5 nmol lipid at a surface of 65 cm^2. The compression and expansion rate was 15 cm^2/min. The reproducibility was better than 0.01 nm^2. The interaction with saccharides was measured by adding the saccharides to the stirred subphase while the lipid layer was expanded to zero surface pressure. The final saccharide concentration was as indicated. After 10 min, the monolayer was compressed and expanded several times to ensure reproducibility.

3. Results

To measure a possible membrane interaction of fructans, monomolecular layers of phospholipids were used as a model membrane. The phospholipids used, exhibited either a phase transition at the air–water interface at ambient temperatures, as dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylethanolamine (DMPE), or a liquid expanded behavior as dioleoylphosphatidylcholine (DOPC).

Membrane interaction of saccharides was measured as the change in surface pressure of DPPC monolayers. At an interface of constant area a lipid monolayer was formed of 20 mN/m and subsequently saccharides were injected into the subphase to the indicated concentration. At the initial pressure of 20 mN/m the DPPC monolayer is in a condensed state (compare Fig. 2). The levan-type fructans produced by *B. subtilis* induced already at low concentrations large changes in film pressure of 20–25 mN/m (Fig. 1). This means that the final film pressure is

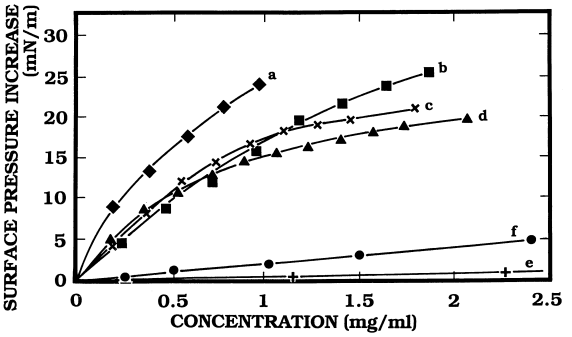

Fig. 1. Change in surface pressure after the injection of increasing mounts of fructans underneath a monomolecular layer of DPPC. The initial surface pressure was 20 mN/m. (a) Total mixture of fructans (isolated by method A) (♦). Fructans isolated and separated by method B: (b) fraction with DP <150 (■) (short); (c) fraction with DP >1500 (×) (long); (d) fraction with DP ~ 300 (▲) (intermediary); (e) inulin-type fructans from Meioligo with DP 3–5 (+); and (f) inulin-type fructans from *H. tuberosus* (●).

40–45 mN/m. The highest pressure increases were observed for the total fructan mixture, containing very high molecular weight polymers. The FPLC purified and separated polymer fractions (long, intermediate and short) showed about 25% lower pressure changes. The effects are measured at concentrations of 0.5–2 mg/ml which is in the range found in fructan accumulating plants. Fructans with a low degree of polymerization and fructan of the inulin-type from *H. tuberosus* had relatively little effect (Fig. 1).

Of the mono- and di-saccharides, fructose and glucose gave relatively small effects, at much higher concentrations. At a concentration of 10–20 mg/ml, pressure increases of 3–4.5 mN/m could be measured. This was the maximal pressure increase obtained for the monosaccharides. Trehalose and sucrose did not show a saturation pressure at that concentration range. Even at a concentration of 50 mg/ml (130 mM) the pressure change induced by trehalose was not maximal and reached 6 mN/m (data not shown).

In order to obtain more insight into the effect of fructans on membrane stability, its potency to influence the phase transition in lipid monolayers was tested. Compression curves of dipalmitoylphosphatidyl-choline (DPPC) and dipalmitoylphosphatidyl-etanolamine (DMPE) were measured in the absence and in the presence of saccharide in the subphase. Equilibration was allowed for 10 min at high surface area before compression. The films were re-expanded

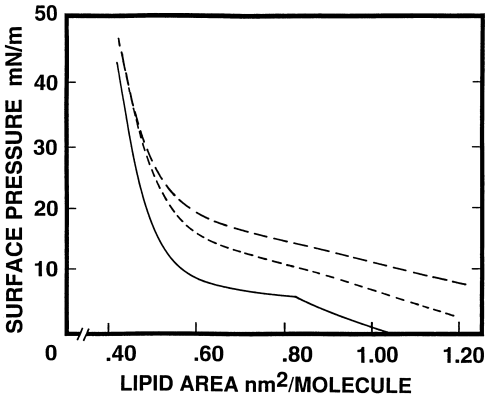

Fig. 2. Pressure–area curve of DPPC in the absence (———) or presence of 0.17 mg/ml (- -) or 0.33 mg/ml (—— ——) total fructan fraction obtained from *B. subtilis*. Equilibration was allowed for 10 min at high surface area before compression.

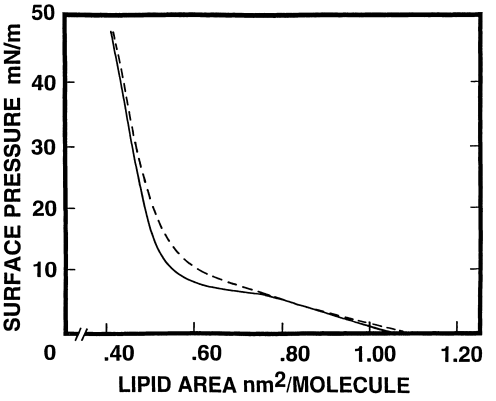

Fig. 3. Pressure–area curve of DPPC in the absence (———) or presence of 4.7 mg/ml (- -) trehalose in the subphase. Equilibration was allowed for 10 min at high surface area before compression.

and compressed several times to test the stability. The pressure–area curves remained completely reproducible.

In agreement with data in the literature DPPC showed a phase transition from a liquid-expanded to condensed phase at 5 mN/m at 21°C.

The levan-type fructans produced by *B. subtilis* had a large effect on the phase transition of DPPC (Fig. 2). At very low fructan concentrations of 0.17 mg/ml, the phase transition was eliminated and the pressure–area curve largely expanded at surface pressures <20 mN/m. However, also at surface pressures >20 mN/m, there was an expansion of the pressure–area curve indicating an interaction of the fructans with the lipid interface. Increasing the fructan concentration to 0.33 mg/ml did lead to a further expansion in the lower pressure region but not in the higher pressure region, indicating that a saturating concentration is reached in the interaction with DPPC, under these conditions.

In the presence of 4.7 mg/ml (12 mM) trehalose, the DPPC pressure–area curve had shifted to larger molecular area in the transition region. The effects on the liquid expanded region between 0.1 and 5 mN/m and at the condensed region at pressures >30 mN/m were very small (Fig. 3).

A 3.9 mg/ml (11 mM) amount of sucrose had a much smaller effect on the phase transition region of DPPC and no measurable effect on the low and high pressure region of the compression curve (data not shown).

The above experiments were also performed with DMPE. The transition point of DMPE from a liquid expanded to condensed phase was found at 7.4 mN/m at 21°C. The levan-type fructans produced by *B. subtilis* also increased the film pressure of the DMPE monolayer both in the expanded and condensed state. However, the transition point was not completely eliminated even at a concentration of 1.28 mg/ml. Increasing concentrations of fructan caused a nearly linear shift in the pressure–area curve (Fig. 4). Probably different amounts of fructan are interacting with either DPPC or DMPE.

Trehalose eliminated the phase transition of DMPE at concentrations of 11.8 mg/ml (30 mM),

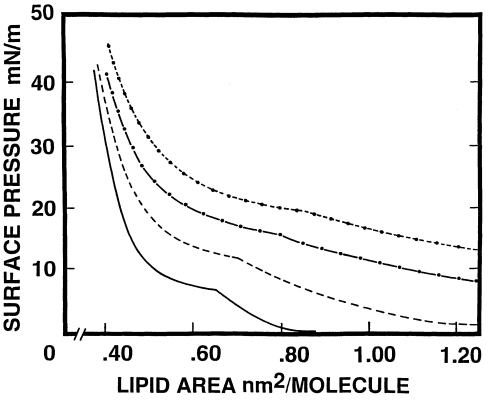

Fig. 4. Pressure–area curve of DMPE in the absence (———) or presence of 0.36 mg/ml (- -); 0.78 mg/ml (– • –) or 1.28 mg/ml (- - • - -) total fructan fraction obtained from *B. subtilis*. Equilibration was allowed for 10 min at high surface area before compression.

but had essentially no effect on the pressure–area curve at pressures <7.4 and >20 mN/m (data not shown). Sucrose at concentrations of 3.9 and 7.8 mg/ml had no measurable effect on the pressure–area curve of DMPE the phase transition was unaffected.

Monolayers of DOPC are liquid-expanded over the entire pressure–area curve due to the presence of a *cis* double bond in the acyl chains [5]. At a surface pressure of 30 mN/m the molecular area of DOPC was significantly enlarged compared to DPPC or DMPE at this pressure. This involves a larger spacing between the polar head groups with the possibility to accommodate more saccharide. Although monomolecular layers of DOPC were affected by levan-type fructan, it was to a lesser extent than found for DPPC or DMPE monolayers. At higher surface pressures (>30 mN/m), the effect was very small (data not shown).

In order to establish that the observed effects are not caused by surface activity of the saccharide or surface active impurities, the surface tension effects of saccharides were measured in the absence of a lipid monolayer.

Trehalose, sucrose, fructose and glucose at concentrations of 20 mg/ml (50–100 mM) had little effect on the surface tension of water. The changes were less than 0.6 mN/m.

The fructans from *H. tuberosus* and from dahlia showed maximal changes in surface tension of 0.8 mN/m. Due to the poor solubility of these fructans of the inulin-type the maximal concentration that could be tested was 2 mg/ml. The fructans of the inulin-type obtained from Meioligo showed a higher water solubility due to the low degree of polymerization. At a concentration of 2 mg/ml, the effect on the surface tension of water was less than 0.1 mN/m. At a concentration of 10 mg/ml the effect had increased to 1.2 mN/m. The fructans of the levan-type produced by *B. subtilis*, which consist of a range of fructans of high polymerization degree, showed little or no surface activity at concentrations <0.5 mg/ml. At concentrations >0.5 mg/ml there was a large and increasing effect on the surface tension. The total mixture showed a surface pressure of 15 mN/m at a concentration of 1.5 mg/ml. At 3 mg/ml, the effect reached a maximum of 20 mN/m.

It can be excluded that the effect was caused by surface active contaminants. Purified and fractionated fructans of *B. subtilis* on Sephacryl S200-HR as described in method B (Section 2.1.2), showed essentially the same effects as the total fructan fraction extracted by method A. Furthermore, repeated compression and decompression of the monolayer did not lead to a change in the pressure–area curve, while surface-active contaminants can be expected to be expelled at high surface pressures and cause a shift at recompression. Degradation of the fructans into smaller units by β-fructosidase to prove additionally the absence of contaminants was not feasible due to the high surface activity of the enzyme itself. It is concluded that the surface active effect is an intrinsic property of the *B. subtilis* synthesized fructans.

4. Discussion

The role of saccharides in the protection against harmful effects of drought has been extensively studied over the last decade. Of the saccharides studied, mainly mono- and di-saccharides, trehalose and sucrose were found to be the most competent in restraining membrane damage during desiccation [6,7]. Another carbohydrate that was discovered in relation with drought resistance in plants is fructan [4]. Dehydration and cooling can cause phase changes in the lipid part of membranes which lead to increased membrane permeability [8–11] and loss of cellular functioning [12,13]. These phase changes can be from a lamellar liquid–crystalline to a lamellar gel phase organization or from a bilayer to a non-bilayer organization. It is assumed that the main action of sugars would be to prevent such harmful phase changes.

In the present study, the possible interaction of fructose polymers with membranes is studied by the use of monomolecular layers. The effect of fructans on phospholipid packing and insertion into the lipid layer can be measured as a change in surface pressure using a trough of constant surface area. Monolayers of DPPC at an initial surface pressure of 20 mN/m are densely packed. When injected into the subphase mono- and di-saccharides show little effect on the surface pressure and also levan-type fructans of a low polymerization degree (DP 2–3) as well as fructans of the inulin-type had little effect.

On the other hand, levan-type fructans of a high degree of polymerization showed large effects on the surface pressure with increases of up to 25 mN/m. This means that the final pressure reaches 45 mN/m which equals the collapse pressure of most membrane lipids. This implies the pressure increases must be due to fructan–lipid interactions and are not caused by the surface activity of the levan-type fructan.

In the interaction of saccharides with phospholipids the type of saccharide, the degree of polymerization and the type of linkage are critical for the interfacial organization and interaction with the lipid phase. It is believed that particularly the prevention of membrane phase transitions is important in the survival of stress conditions of plants. Lipid phase transitions in monolayers at the air–water interface were measured by the compression of a defined amount of a particular lipid defined by acyl chain lengths and polar head group. DPPC and DMPE show a phase transition at ambient temperatures. The shorter chain length of DMPE, compared to DPPC, is compensated by the increased head group interaction. Compression curves in the absence and in the presence of saccharides demonstrate the effect on the phase transition. The present study shows large interfacial effects for levan-type fructans at very low concentrations. The phase transition of DPPC is eliminated and of DMPE is hardly visible whereas the monolayer has expanded also at high surface pressures (>30 mN/m). Repeated expansion to surface pressures <1 mN/m and compression to a surface pressure of 45 mN/m of the monolayer did not change the pressure–area curve. This indicates a stable interfacial interaction of lipid and fructan and refute that the effects can be simply explained by the surface activity of the fructan. There is a remarkable difference between the fructan effects on DPPC and DMPE. Although the effect on DPPC seems to saturate at high pressures, increasing amounts seem to bind to DMPE. A possible explanation could be the interference of fructan in the polar head group interactions of the phospholipid.

Of the disaccharides, trehalose had an effect that was practically restricted to the phase transition region of DPPC and DMPE whereas for sucrose this was only measurable for the DPPC monolayer. It seems that the interaction of disaccharides with DMPE is reduced compared to DPPC. The observations equate to those of Arnett et al. [14] who found hardly any differences in the liquid-expanded pressure–area curves of dimyristoylphosphatidylcholine measured in the presence or absence of trehalose and calorimetry studies on aqueous dispersions [15]. Results obtained with carbohydrates and dry dipalmitoylphosphatidylcholine using IR spectroscopy suggested that the mechanism of interaction involves hydrogen bonding between carbohydrate and the phosphate head group of the phospholipid [16]. It is believed that the sugar replaces water and maintains the lateral spacing between lipid polar head groups in the dry state, thereby minimizing van der Waals interactions of the hydrocarbon chains. Sugars have the ability to form glasses, which have very high viscosity and low mobility. Koster et al. [17] reported that the glass transition temperature for the dry sugar is decisive in depressing the transition temperature in the dry lipid. The predictions of the vitrification hypothesis seem to be fulfilled for low molecular weight molecules, but breaks down above the trisaccharide level [18]. Until recently, it was assumed that glucoside chains longer than tri-glucoside show less cryoprotective effects than glucoside chains of mono-, di- and tri-glucosides [19]. However, Ozaki and Hayashi [20] showed for cycloinulohexaose a cryoprotective effect on freezing and freeze-drying of liposomes similar to that of trehalose. This cyclohexaose of β-D-fructofranose consisting of β $(2\rightarrow1)$-linked oligofructose chains increased the lipid membrane fluidity in a dry state which prevents leakage during the rehydration process. In this study, we show that levan-type fructan of high DP is effective in lipid interactions. The effects of fructan as demonstrated in monomolecular layers are much stronger than the effects of disaccharides. The concentrations required are an order of magnitude lower for fructan than for disaccharide and similar to that found in fructan accumulating plants.

The special properties of levan-type fructan might originate from the fructosyl residues and the glycosidic bond which is β $(2\rightarrow1)$ and β $(2\rightarrow6)$. Fructan has a high solubility which could indicate that hydroxyl groups are largely available for interactions with surrounding water molecules and membrane phospholipid head groups when present. The special sidedness of hydroxyl and hydrogen moieties might

be responsible for the high interfacial affinity of furan and their ability to insert into phospholipid membranes. Another advantageous property could be the readily interconversion between conformational states of furanose rings [21]. The demonstrated membrane interaction of fructan which prevents lipid condensation and phase transitions to arise is likely to induce water-stress protection.

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