Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1612 (2003) 172–177
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions

Dirk K. Hincha*, Ellen Zuther, Arnd G. Heyer

Max-Planck-Institut für Molekulare Pflanzenphysiologie, D-14424 Potsdam, Germany

Received 1 October 2002; received in revised form 26 March 2003; accepted 4 April 2003

Abstract

Raffinose family oligosaccharides (RFO) have been implicated as protective agents in the cellular dehydration tolerance, especially of many plant seeds. However, their efficacy in stabilizing membranes during dehydration has never been systematically investigated. We have analyzed the effects of sucrose, raffinose, stachyose, and verbascose on liposome stability during air-drying. With increasing degree of polymerization (DP), the RFO were progressively better able to stabilize liposomes against leakage of aqueous content and against membrane fusion after rehydration. Indeed, there was a very tight linear correlation between fusion and leakage for all RFO. These data indicate that increased protection of liposomes against leakage with increasing DP is due to better protection against fusion. This is in accord with the higher glass transition temperature of the longer chain oligosaccharides. Further evidence for the influence of glass transitions on membrane stability in the dry state was provided by experiments testing the temperature dependence of membrane fusion. During incubation at temperatures up to 95 °C for 2 h, fusion increased less with temperature in the presence of higher DP sugars. This indicates that RFO with a higher glass transition temperature are better able to protect dry membranes at elevated temperatures. In addition, Fourier-transform infrared (FTIR) spectroscopy showed a reduction of the gel to liquid-crystalline phase transition temperature of dry liposomes in the presence of all investigated sugars. However, the RFO became slightly less effective with increasing chain length, again pointing to a decisive role for preventing fusion. A direct interaction of the RFO with the lipids was indicated by a strong effect of the sugars on the phosphate asymmetric stretch region of the infrared spectrum.

Keywords: Desiccation; Fourier-transform infrared spectroscopy; Liposome; Raffinose family oligosaccharide

1. Introduction

Raffinose family oligosaccharides are a group of oligosaccharides that are synthesized by many plant species (see Ref. [1] for a recent review). They are derivatives of sucrose. Galactose is added to the glucose part of sucrose through an α(1,6) bond (raffinose). Further galactose units are added through α(1,6) bonds to the terminal galactose to form the tetra- and pentasaccharides stachyose and verbascose, respectively. RFO are accumulated in the seeds of many species during the late stages of development, coincident with the development of desiccation tolerance [2,3]. Further indirect evidence for a role of RFO in cellular stress tolerance is provided by the fact that some species also accumulate RFO in their leaves during cold acclimation [4–9]. Recently, it was shown that transgenic *Arabidopsis thaliana* plants that overexpress a galactinol synthase gene, encoding the first enzyme in the RFO biosynthetic pathway, accumulate raffinose and become more desiccation tolerant than the wild type [10].

In seeds, vitrification of the cytoplasm during drying is considered to be a crucial aspect of desiccation tolerance [11,12]. The same has been suggested for the dry leaves of the resurrection plant *Craterostigma plantagineum* [13]. Cytoplasmic glasses contain both sugars and proteins [14,15]. Although the glass transition temperature (T_g) of RFO increases with increasing DP from 69 °C for sucrose to 106 °C for raffinose and 123 °C for stachyose [14], which would indicate superior vitrification properties for the higher DP RFO, their functional role for glass formation in seeds is not clear [14,15].

Since membranes are the primary targets of both freezing and desiccation injury in cells [16–19], a role of RFO

* Corresponding author. Tel.: +49-331-567-8253; fax: +49-331-567-8250.
E-mail address: hincha@mpimp-golm.mpg.de (D.K. Hincha).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00116-0

in cellular stress tolerance could, in addition to glass formation, also involve the direct stabilization of membranes under stress conditions. Membrane stabilization has been shown for raffinose during freeze-drying of microsomes from lobster muscle [20], for protoplasts isolated from desiccation-tolerant pea seed embryos [21], and for raffinose and stachyose during freezing of spinach thylakoids [22].

With liposomes, it has been shown that oligosaccharides of different chain length can significantly vary in their ability to stabilize membranes. Malto-oligosaccharides lose their ability to interact with membrane lipids in the dry state with increasing DP, while fructo-oligosaccharides (inulins) show increased interaction [23]. We show here that, with increasing DP, RFO reduced leakage of a soluble marker and afforded better protection against membrane fusion in liposomes. This was especially pronounced when dry samples were incubated at elevated temperatures. Under these conditions, the higher glass transition temperature of the longer chain RFO led to reduced fusion of the liposomes. Using Fourier-transform infrared (FTIR) spectroscopy, we were able to show that RFO of all DP interact with membrane lipids in the dry state and reduce T_m.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, AL) or Sigma. Carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, OR) and was purified according to the procedure described in Ref. [24]. *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Rh-PE) were purchased from Molecular Probes. Sucrose, raffinose, and stachyose were purchased from Sigma, verbascose was obtained from Gammazym (Gamma Chemie, München, Germany).

2.2. Sugar analysis

Sugars were analyzed by high performance liquid anion exchange chromatography (HPAEC) using a CarboPac PA-100 column on the Dionex DX-500 gradient chromatography system (Dionex, Sunnyvale, CA) coupled with pulsed amperometric detection by a gold electrode. The column was equilibrated in 0.05 M NaOH and was eluted with a linear gradient from 0.05 M to 0.2 M NaOH.

2.3. Preparation of liposomes

EPC was dried from chloroform under a stream of N_2 and stored under vacuum overnight to remove traces of solvent. Liposomes were prepared from hydrated lipids using a hand-held extruder with two layers of polycarbonate membranes with 100-nm pores (Avestin, Ottawa, Canada) [25].

2.4. Leakage experiments

For leakage experiments, an appropriate amount of lipid was hydrated in 0.25 ml of 100 mM CF, 10 mM TES, 0.1 mM EDTA (pH 7.4). After extrusion, the vesicles were passed through a NAP-5 column (Sephadex G-25; Pharmacia) equilibrated in TEN buffer (10 mM TES, 0.1 mM EDTA (pH 7.4), 50 mM NaCl), to remove the CF not entrapped by the vesicles. The eluted samples were then diluted with TEN to a lipid concentration of approximately 10 mg ml^{-1}. Liposomes (40 μl) were mixed with an equal volume of concentrated solutions of sugars in TEN and 20-μl aliquots were filled into the wells of 60-well microplates. The plates were dried in desiccators at 28 °C and 0% relative humidity for 24 h in the dark. Damage to the liposomes was determined as CF leakage after rehydration as described in detail in a recent publication [23]. The figures show the means ± S.D. from three parallel samples. Where no error bars are visible, they are smaller than the symbols.

2.5. Fusion experiments

For liposome fusion experiments, two liposome samples were prepared in TEN. One contained 1 mol% each of

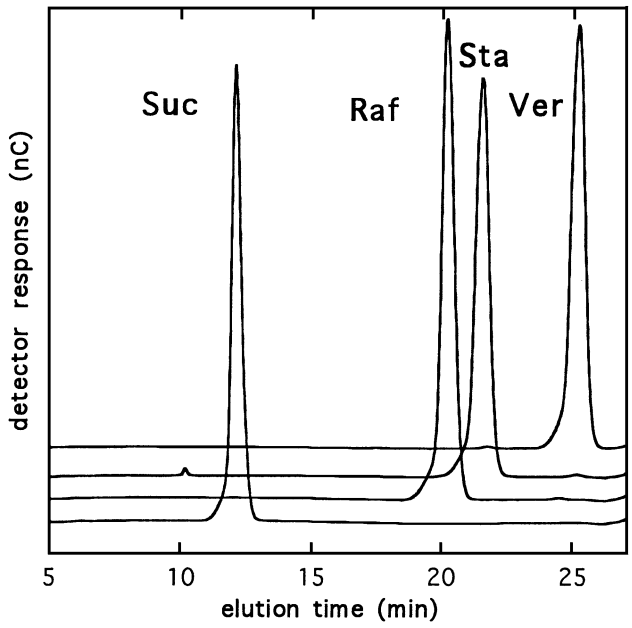

Fig. 1. HPLC analysis of the sugars used in the experiments reported in this paper. The sugars sucrose (Suc), raffinose (Raf), stachyose (Sta), and verbascose (Ver) were analyzed by anion exchange chromatography. Longer elution times indicate a higher degree of polymerization. The plots of the different elution profiles were offset for greater clarity.

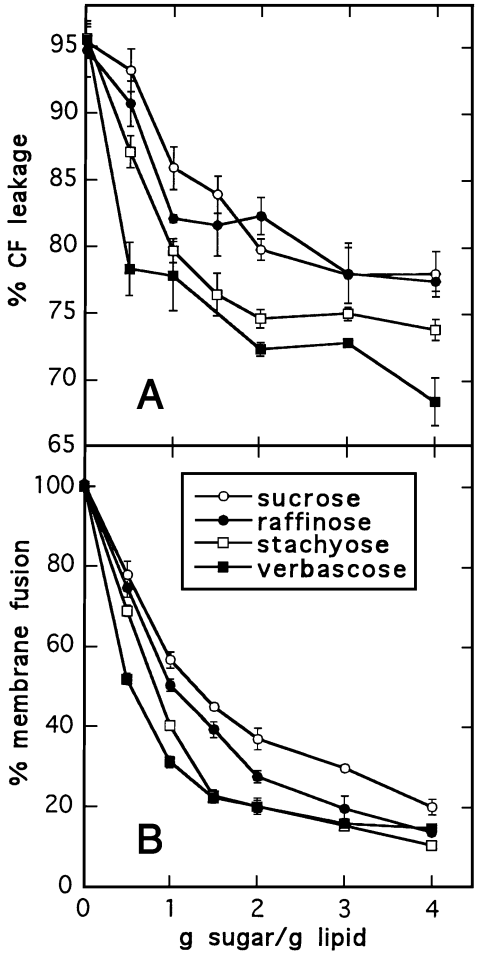

Fig. 2. Protection of large unilamellar liposomes from damage during drying by different sugars. Leakage of carboxyfluorescein (CF) from the vesicles (A) or membrane fusion (B) were determined after air-drying and rehydration. The samples contained the indicated concentrations of sugars in the incubation solution. In addition, the vesicles in (A) also encapsulated 4 g of the respective sugars per gram of lipid.

NBD-PE and Rh-PE in EPC, the other contained only EPC. After extrusion, liposomes were combined at a ratio of 1:9 (labeled/unlabeled), resulting in a lipid concentration of 10 mg ml^{-1}. Liposomes (40 μl) were mixed with an equal volume of concentrated solutions of sugars in TEN and 20-μl aliquots were filled into the inside of the caps of 1.5-ml microcentrifuge tubes. Samples were dried as described above. In some cases, the desiccators with the dry samples were subsequently stored in a water bath at higher temperatures for 2 h. Measurements with a fine thermocouple showed that it took approximately 30 min for the samples to equilibrate at these temperatures. The samples were rehydrated by filling 1 ml of TEN buffer into a tube, and then quickly closing and inverting the tube. Membrane fusion was measured by resonance energy transfer [26] as described [27,28]. The figures show the means ± S.D. from three parallel samples. Where no error bars are visible, they are smaller than the symbols.

2.6. FTIR spectroscopy

Spectra were obtained from samples containing EPC liposomes and sugar at a weight ratio of 1:2. Liposomes were extruded in the presence of the sugars, so that the sugars were present on both sides of the membranes. Samples (50 μl) were spread on CaF_2 windows and dried under the same conditions as described above. A window was then fixed in the vacuum chamber of a cuvette holder connected to a temperature control unit (Specac Eurotherm, Worthington, UK) [23]. The sample was first heated to 50 °C for 20 min under vacuum to remove residual moisture the lipid had taken up during sample handling. The effectiveness of this procedure was verified by the absence of a water band in the FTIR spectra at 1650 cm^{-1}. The sample was then cooled to −30 °C and after a 20-min equilibration, the temperature was increased at a constant rate of 1 °C min^{-1}. Spectra were recorded with a Perkin-Elmer GX 2000 FTIR spectrometer. After normalization of absorbance using the interactive abex routine, the peak frequencies of the CH_2 symmetric stretch band around 2850 cm^{-1} were determined by the automatic peak identification routine. T_m values were estimated as the midpoints of the lipid melting curves [29]. The phosphate asymmetric stretch vibrations in the 1300–1200 cm^{-1} region were analyzed after normalization of absorbance and baseline flattening, using the interactive abex and flat routines, respectively [30].

3. Results and discussion

For a meaningful comparison of the effects of different RFO on membrane stability during drying, we first estab-

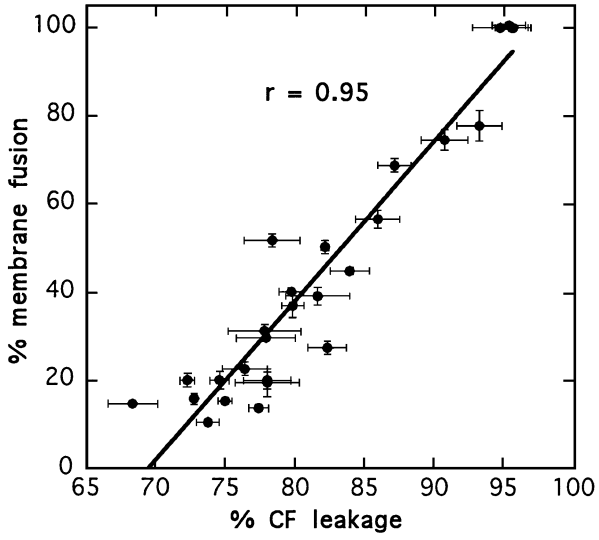

Fig. 3. Correlation between CF leakage and membrane fusion after drying of samples containing different concentrations of the RFO as shown in Fig. 2. The straight line was fitted to the data by linear regression analysis and the correlation coefficient is shown.

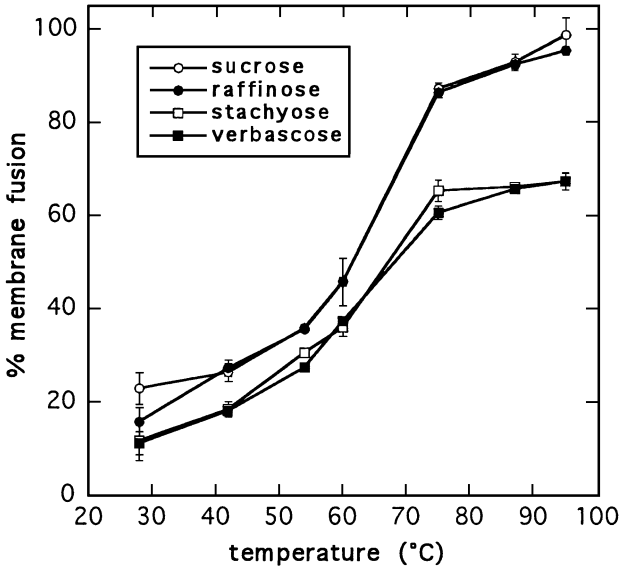

Fig. 4. Temperature dependence of the stability of liposomes in the dry state. Samples containing 4 g of the indicated sugars per gram of lipid in the incubation solution were dried at 28 °C for 24 h. They were then incubated for an additional 2 h at the indicated temperatures. Samples were rehydrated with buffer at room temperature (approximately 22 °C) and the degree of membrane fusion was determined.

lished the purity of the sugars. We used analytical HPLC to analyze the chain-length distribution in the sugar preparations used in the subsequent experiments. Fig. 1 shows that all preparations contained the correct sugar with a purity of at least 95%. Especially, there were no mono- and disaccharide contaminants in the higher degree of polymerization (DP) RFO.

All sugars had a stabilizing effect on EPC liposomes after drying and rehydration (Fig. 2). The RFO showed increased protection against CF leakage with increasing DP (Fig. 2A). In these experiments, the liposomes were loaded with 4 g of sugar per gram of lipid. Protection by the corresponding external sugar was measured after drying and rehydration. It has been shown before that the presence of sugars inside of liposomes increases the protection against CF leakage afforded by external sugars [23,31]. Protection against membrane fusion (Fig. 2B) was measured in samples that only contained external sugars, as fusion would not be expected to be influenced by internal sugars. A similar trend as for the leakage measurements of increased protection with increasing DP was also apparent from the fusion measurements. In the concentration range used, the effects on fusion were much stronger than the effects on leakage. This is in agreement with previous findings [23,32].

Fig. 3 shows that there was indeed a very tight correlation between fusion and leakage for all investigated samples. This indicates that the different effectiveness of the RFO in preventing leakage was mainly due to their different abilities to prevent fusion.

Protection of membranes against fusion by sugars has been related to the ability of sugars to form glasses (vitrify) during drying [33]. The propensity of oligomeric substances to vitrify generally increases with increasing DP [34,35]. For the RFO this is reflected in the increased T_g with increasing DP [14]. Therefore, the results presented in Fig. 2B are not unexpected, although we have recently shown that fructo-oligosaccharides are less effective in preventing fusion with increasing DP [23].

It has been shown earlier that fusion and leakage in liposomes contained in a vitrified sugar matrix are strongly temperature dependent, especially when the incubation temperature approaches the glass transition temperature of the respective sugar [36]. The RFO used in our study have

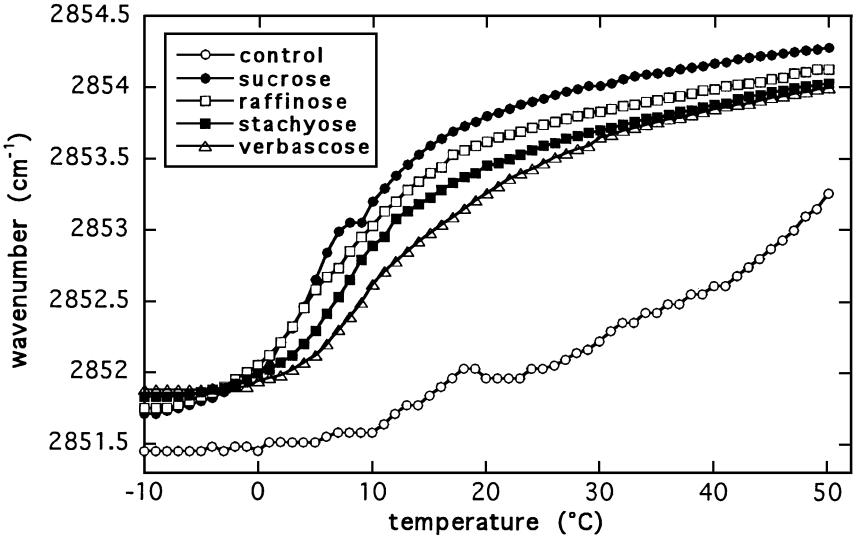

Fig. 5. Lipid melting curves of dry EPC liposomes as determined by FTIR spectroscopy. The wave number of the CH_2 symmetric stretch peak is plotted as a function of the sample temperature. T_m was determined as the midpoint of each melting curve. The samples contained either only EPC liposomes without additional sugars (T_m = 40 °C), or EPC liposomes with sucrose (T_m = 7 °C), raffinose (T_m = 9 °C), stachyose (T_m = 11 °C), or verbascose (T_m = 13 °C) at a 1:2 mass ratio both inside and outside of the liposomes.

T_g's from 69 °C (sucrose) to at least 123 °C (stachyose; no data available for verbascose). Therefore, we incubated dry samples in the presence of a high concentration of these sugars at temperatures up to 95 °C to investigate whether melting of the glasses might differentially influence the stability of the membranes in the presence of the different sugars. It is apparent from Fig. 4 that elevated temperatures led to increased membrane fusion in the presence of all investigated sugars. However, the difference between the sugars increased at the higher temperatures. For instance, the difference in fusion between sucrose and verbascose was 11.8% at 28 °C and 31.4% at 95 °C. These data clearly indicate that the higher T_g sugars provide superior protection to the liposomes at elevated temperatures.

CF leakage during drying and rehydration is, in addition to vesicle fusion, also due to gel to liquid-crystalline phase transitions of the membrane lipids (reviewed in Refs. [18,37]). To gain further insight into the physical mechanisms underlying the membrane stabilization by RFO, we used FTIR spectroscopy. We determined the gel to liquid-crystalline lipid phase transition temperature (T_m) of the dried EPC liposomes by monitoring the frequency of the CH_2 symmetric stretching mode around 2850 cm^{-1}, which increases by 2–3 wavenumbers as the chains melt [38]. Fig. 5 shows that in the presence of the different sugars, T_m (determined as the midpoint of the transitions) of dry EPC was strongly reduced. The dry lipid in the absence of additional sugars had a T_m of 40 °C, which is in good agreement with published data [23,29]. In the presence of sucrose, T_m was 7 °C and in the presence of raffinose, T_m was about 9 °C, while it was 11 °C with stachyose and 13 °C with verbascose. These results are in agreement with previously published data [39] showing T_m values for dry dipalmitoyl-PC that were similar in the presence of sucrose, raffinose, and stachyose after equilibration in the gel phase.

It has been suggested that the reduction in T_m observed in dry lipids in the presence of sugars is due to a direct hydrogen bonding interaction between the sugars and the phospholipid headgroups (reviewed in Refs. [18,37]). This interaction can be detected as a shift in the frequency of the asymmetric phosphate vibration in the FTIR spectra around 1240 cm^{-1}. Fig. 6 shows such spectra from dry EPC liposomes in the absence of additional sugars and in the presence of sucrose and RFO. The EPC control samples showed a peak at 1259 cm^{-1}. This was shifted by about 20 wavenumbers in the presence of sucrose. The RFO showed a reduction of this shift with increasing DP. Nevertheless, there was a clear indication of hydrogen bonding in all cases, in accordance with the reduction in T_m.

Plants can accumulate substantial amounts of RFO, both in seeds that acquire desiccation tolerance [2] and in leaves that acclimate to cold or drought stress [4–9]. Therefore, the RFO concentrations we have used in our experiments are expected to be present in plant cells. We conclude from our results that RFO of all physiologically relevant DPs may, in addition to their ability to form glasses, also be involved in the stabilization of cellular membranes under stress conditions by direct sugar–membrane interactions that could contribute to the beneficial effects of these sugars [40,41].

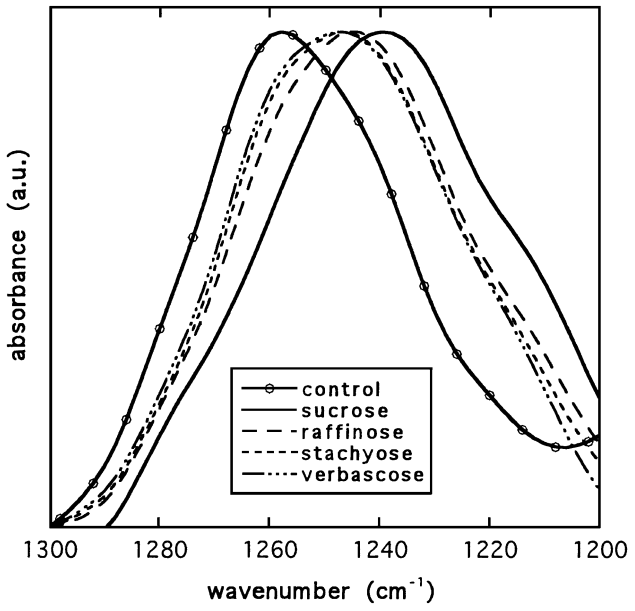

Fig. 6. FTIR spectra of the phosphate asymmetric stretch region in dry EPC liposomes. The samples contained either only EPC liposomes without additional sugars (control), or EPC liposomes and sucrose, raffinose, stachyose, or verbascose at a 1:2 mass ratio both inside and outside of the liposomes. Spectra were recorded at 50 °C to ensure that the membranes were in the liquid-crystalline phase in all cases (compare Fig. 5; a.u. = arbitrary units). The peak positions are: 1259.0 cm^{-1} (control); 1238.8 cm^{-1} (sucrose); 1244.0 cm^{-1} (raffinose); 1246.4 cm^{-1} (stachyose); 1247.5 cm^{-1} (verbascose).

References

[1] T. Peterbauer, A. Richter, Seed Sci. Res. 11 (2001) 185–197.
[2] T.M. Kuo, J.F. VanMiddlesworth, W.J. Wolf, J. Agric. Food Chem. 36 (1988) 32–36.
[3] R.L. Obendorf, Seed Sci. Res. 7 (1997) 63–74.
[4] M. Bachmann, P. Matile, F. Keller, Plant Physiol. 105 (1994) 1335–1345.
[5] Y. Castonguay, P. Nadeau, P. Lechasseur, L. Chouinard, Crop Sci. 35 (1995) 509–516.
[6] Y. Castonguay, P. Nadeau, Crop Sci. 38 (1998) 1183–1189.
[7] S.J. Gilmour, A.M. Sebolt, M.P. Salazar, J.D. Everard, M.F. Thomashow, Plant Physiol. 124 (2000) 1854–1865.
[8] K.L. Koster, D.V. Lynch, Plant Physiol. 98 (1992) 108–113.
[9] K.A. Santarius, H. Milde, Planta 136 (1977) 163–166.
[10] T. Taji, C. Ohsumi, S. Iuchi, M. Seki, M. Kasuga, M. Kobayashi, K. Yamaguchi-Shinozaki, K. Shinozaki, Plant J. 29 (2002) 417–426.
[11] J. Buitink, M.A. Hemminga, F.A. Hoekstra, Plant Physiol. 122 (2000) 1217–1224.
[12] R.J. Williams, A.C. Leopold, Plant Physiol. 89 (1989) 977–981.
[13] W.F. Wolkers, H. Oldenhof, M. Alberda, F.A. Hoekstra, Biochim. Biophys. Acta 1379 (1998) 83–96.
[14] J. Buitink, I.J. van den Dries, F.A. Hoekstra, M. Alberda, M.A. Hemminga, Biophys. J. 79 (2000) 1119–1128.
[15] W.F. Wolkers, M. Alberda, M. Koorneef, K.M. Leon-Kloosterziel, F.A. Hoekstra, Plant J. 16 (1998) 133–143.

[16] J.H. Crowe, F.A. Hoekstra, L.M. Crowe, Annu. Rev. Physiol. 54 (1992) 579–599.
[17] D.K. Hincha, F. Sieg, I. Bakaltcheva, H. Köth, J.M. Schmitt, in: P.L. Steponkus (Ed.), Advances in Low-Temperature Biology, vol. 3, JAI Press, London, 1996, pp. 141–183.
[18] A.E. Oliver, D.K. Hincha, J.H. Crowe, Comp. Biochem. Physiol. 131A (2002) 515–525.
[19] P.L. Steponkus, Annu. Rev. Plant Physiol. 35 (1984) 543–584.
[20] L.M. Crowe, R. Mouradian, J.H. Crowe, S.A. Jackson, C. Womersley, Biochim. Biophys. Acta 769 (1984) 141–150.
[21] L. Xiao, K.L. Koster, J. Exp. Bot. 52 (2001) 2105–2114.
[22] D.K. Hincha, CyroLetters 11 (1990) 437–444.
[23] D.K. Hincha, E. Zuther, E.M. Hellwege, A.G. Heyer, Glycobiology 12 (2002) 103–110.
[24] J.N. Weinstein, E. Ralston, L.D. Leserman, R.D. Klausner, P. Dragsten, P. Henkart, R. Blumenthal, in: G. Gregoriadis (Ed.), Liposome Technology, vol. 3, CRC Press, Boca Raton, FL, 1984, pp. 183–204.
[25] R.C. MacDonald, R.I. MacDonald, B.P.M. Menco, K. Takeshita, N.K. Subbarao, L. Hu, Biochim. Biophys. Acta 1061 (1991) 297–303.
[26] D.K. Struck, D. Hoekstra, R.E. Pagano, Biochemistry 20 (1981) 4093–4099.
[27] A.E. Oliver, D.K. Hincha, L.M. Crowe, J.H. Crowe, Biochim. Biophys. Acta 1370 (1998) 87–97.
[28] D.K. Hincha, A.E. Oliver, J.H. Crowe, Biochim. Biophys. Acta 1368 (1998) 150–160.
[29] J.H. Crowe, A.E. Oliver, F.A. Hoekstra, L.M. Crowe, Cryobiology 35 (1997) 20–30.
[30] N.M. Tsvetkova, B.L. Phillips, L.M. Crowe, J.H. Crowe, S.H. Risbud, Biophys. J. 75 (1998) 2947–2955.
[31] J.H. Crowe, L.M. Crowe, Biochim. Biophys. Acta 939 (1988) 327–334.
[32] L.M. Crowe, J.H. Crowe, A. Rudolph, C. Womersley, L. Appel, Arch. Biochem. Biophys. 242 (1985) 240–247.
[33] J.H. Crowe, J.F. Carpenter, L.M. Crowe, Annu. Rev. Physiol. 60 (1998) 73–103.
[34] H. Levine, L. Slade, CyroLetters 9 (1988) 21–63.
[35] L. Slade, H. Levine, Crit. Rev. Food Sci. Technol. 30 (1991) 115–360.
[36] W.Q. Sun, A.C. Leopold, L.M. Crowe, J.H. Crowe, Biophys. J. 70 (1996) 1769–1776.
[37] A.E. Oliver, L.M. Crowe, J.H. Crowe, Seed Sci. Res. 8 (1998) 211–221.
[38] H.H. Mantsch, R.N. McElhaney, Chem. Phys. Lipids 57 (1991) 213–226.
[39] J.H. Crowe, F.A. Hoekstra, K.H.N. Nguyen, L.M. Crowe, Biochim. Biophys. Acta 1280 (1996) 187–196.
[40] P. Brenac, M. Horbowicz, S.M. Downer, A.M. Dickerman, M.E. Smith, R.L. Obendorf, J. Plant Physiol. 150 (1997) 481–488.
[41] W.Q. Sun, A.C. Leopold, Comp. Biochem. Physiol. 117A (1997) 327–333.