Trehalose: Current Use and Future Applications

SATOSHI OHTAKE,[1] Y. JOHN WANG[2]

[1]Aridis Pharmaceuticals, San Jose, California 95138

[2]Late Stage Pharmaceutical and Process Development Department, Genentech, South San Francisco, California 94080

Received 29 September 2010; revised 5 December 2010; accepted 6 December 2010

Published online 18 February 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22458

ABSTRACT: Trehalose, a disaccharide of glucose, has been reported to accumulate in many organisms that can withstand extended periods of inanimation. Since this discovery, the properties of trehalose have been examined extensively to understand its role and abundance in nature. The unique features of this sugar became clearer with each new finding which demonstrated its ability to sustain and preserve a wide array of biological molecules. Trehalose has been used in a variety of research applications and is contained in several commercially available therapeutic products, including Herceptin®, Avastin®, Lucentis®, and Advate®. Currently, there is a growing interest in the use of trehalose in solid dosage formulations, most notably in quick-dissolving tablets. Furthermore, trehalose has found its use in several food and cosmetic products, and new applications capitalizing on its unique properties are being developed and implemented in everyday-use products. As trehalose is an approved ingredient in all major markets, there is no significant barrier to its use. Extensive work with trehalose has been conducted in the three major industries, however with little overlap. Further understanding of the role of trehalose in the various applications may lead to an increase in the number of trehalose-containing products.
 J Pharm Sci 100:2020–2053, 2011

Keywords: trehalose; stability; formulation; excipients; glass; physicochemical; food; cosmetic

INTRODUCTION

Trehalose has been studied for over a hundred years, and the number of publications examining the effects of trehalose continues to grow each year. Despite the abundance of data, our understanding of this unique sugar remains inadequate. Trehalose, to some readers, may be a magical excipient, whereas to others, it is simply another sugar. No matter the bias of the reader, there is no debate about its unique properties.

There have been a number of review articles published on trehalose[1–23] and the topics vary widely from their production to their use in research and industrial applications. The purpose of the current review is not only to update the readers on the recent developments on the use of trehalose, but also to introduce the unique features of trehalose that may be less known to readers outside of Japan. Several scientific articles, published in Japanese, have been reviewed and a summary of their data has been incorporated. The present review examines the practical applications of trehalose not only in pharmaceuticals but also in the field of foods and cosmetics. In addition, a summary of patent applications and information on commercially available products are included. Another aim of the present review is to educate the users of trehalose in its application outside of its normal use. For example, a formulation scientist may already be knowledgeable about the physical properties of trehalose as they pertain to protein stabilization, but perhaps not about their applications in the food and cosmetic industries. Research scientists in academia may be knowledgeable about the various methods for synthesizing/purifying trehalose, but they may be intrigued to learn about the recent developments in its large-scale production.

The unique properties of trehalose, which provide the breadth of its use, are described first. Following this description, several mechanisms that have been proposed to explain its effects in stabilizing a wide variety of compounds are given. Next, the natural occurrence of trehalose is discussed. The reader will undoubtedly be surprised to learn about the abundance of this sugar in our environment. In nature, trehalose is synthesized within insects, yeasts, and plants. In fact, their mechanism has been mimicked

Correspondence to: Satoshi Ohtake (Telephone: +408-333-9681; Fax: +408-960-3822; E-mail: ohtakes@aridispharma.com)

Journal of Pharmaceutical Sciences, Vol. 100, 2020–2053 (2011)

in the laboratory to synthesize trehalose on a small scale. Commercially, many, if not all, of these methods are not viable. To address this point, the large-scale production method of trehalose from one of the leading manufacturers will be discussed. With the implementation of the novel production method, the cost of trehalose has been reduced significantly, which may have a profound effect in increasing the number of trehalose-containing applications. The use of trehalose in the three major industries, including pharmaceutical/biotechnology, food, and cosmetic, is then discussed. A summary of regulatory issues and pharmacopoeia specifications is given, and future applications of trehalose conclude the review.

UNIQUE PROPERTIES OF TREHALOSE

Trehalose, also known as α- D-glucopyranosyl α- D-glucopyranoside, is a disaccharide of glucose. Due to the linkage of the two glucopyranose rings occurring at the reducing end of the glycosyl residues (α-carbons), trehalose is a nonreducing sugar and demonstrates exceptional stability;[24,25] trehalose is not easily hydrolyzed by acid and the glycosidic bond is not cleaved by α-glycosidase. Although the α,α form is the isomer commonly referred to as trehalose, α,β and β,β isomers exist in nature and display physical properties that are quite different from those of α,α-trehalose.[26]

Trehalose is typically found in the dihydrate form and is characterized by its low hygroscopicity; water content of the dihydrate crystal remains stable at approximately 9.5% during exposure to relative humidity of up to 92%.[7] The dehydration of the dihydrate crystal occurs at 97°C and the anhydrous crystal melts at 210°C.[27–31] The melting temperature of trehalose can vary widely and this is due to its polymorphic nature, which can exist as anhydrous or dihydrate (α, β, or γ).[29] In certain applications, it may be advantageous to process trehalose, typically in the dihydrate crystalline form, into another form (i.e., anhydrous crystal or amorphous), as all the polymorphs possess different physical properties such as malleability and compressibility.[32] Trehalose is soluble in water (34 g/100 g H_2O at 5°C and 40.6–69 g/100 g H_2O at 20°C),[31,33] although less than sucrose. During various desiccation processes, including freeze drying and spray drying, trehalose readily dries as an amorphous material with high glass transition temperature ($T_g > 100$°C).[28,29,34] The reported values in literature do vary, and this discrepancy is due to the different measurement conditions used (i.e., scanning rate used on differential scanning calorimetry (DSC)) and most importantly the residual water content. The T_g for trehalose at 0.3% residual water content was reported to be 111.3°C by Crowe et al.[35] T_g decreases with water addition due to the plasticizing effect of water (Fig. 1); however, the T_g for trehalose remains higher than that for any other disaccharide at comparable water content.

In Table 1, the physical properties of trehalose are compared with those of sucrose, another disaccharide of identical molecular weight, to highlight the key properties that differentiate this unique sugar in its ability to interact/stabilize a wide range of systems and its application in fields as diverse as foods, cosmetics, and pharmaceuticals, as will be described later in more detail. The wide ranging values reported in the table reflect differences in experimental conditions, thus care must be taken when searching for relevant data to ensure that the experimental conditions are similar to those used by the reader. The stability of trehalose to hydrolysis can be explained by the low energy of its glycosidic bond (<1 kcal/mol).[25,37] In comparison, the glycosidic bond of sucrose is higher in free energy (27 kcal/mol), rendering the disaccharide more susceptible to hydrolysis in the presence of a mild acid, producing glucose and fructose, both of which are strong reducing sugars. In another study, the rate of hydrolysis, without acid catalysis, for trehalose was determined to be significantly lower than that for sucrose; 3.3×10^{-15} s^{-1} and 5×10^{-11} s^{-1}, respectively, at 25°C (as determined by extrapolation from the Arrhenius plot, where E_a was 37.1 and 31.4 kcal/mol for trehalose and sucrose, respectively).[38] The correlation between hydrolysis rate and temperature for the two sugars is shown in Figure 2. Wolfenden and Yuan[38] also reported that these rates do not vary significantly with pH, in the range of 7–12, or with ionic strength, in the range of 0.2–2 M KCl. Furthermore, sucrose has been reported to undergo hydrolysis during freezing and

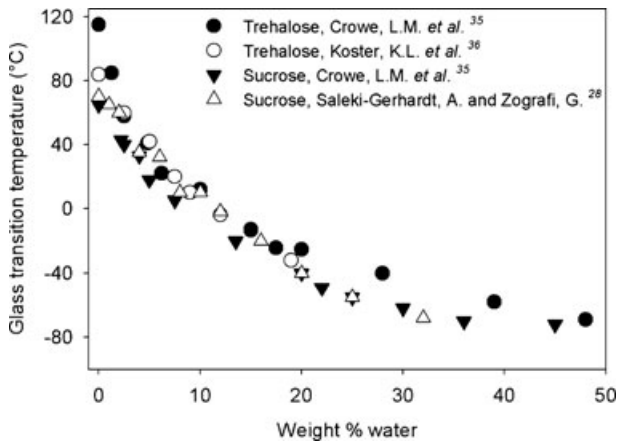

Figure 1. Correlation between glass transition temperature (T_g) and water content (wt %) for trehalose and sucrose. Reproduced from Saleki-Gerhardt and Zografi[28] with permission from Springer, Crowe et al.[35] with permission from Cell Press, and Koster et al.[36] with permission from Cell Press.

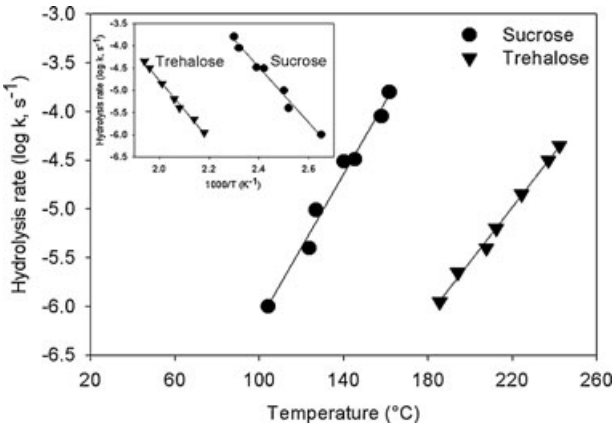

Figure 2. Rate of hydrolysis of disaccharides, trehalose and sucrose, at 0.05 M in 0.1 M potassium phosphate buffer (pH 8.1) at temperatures ranging from 100°C to 240°C. Similar plot in Arrhenius format is shown in the inset. Reproduced from Wolfenden and Yuan[38] with permission from ACS Publication.

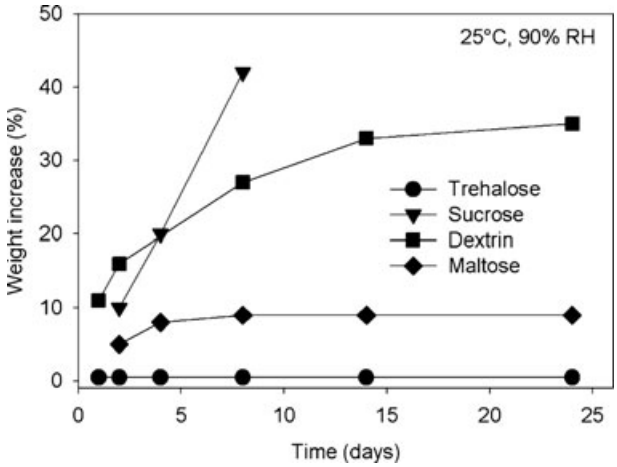

Figure 3. Water uptake of various sugars in the crystalline form following storage at 25°C and 90% relative humidity (RH). Reproduced from Takeuchi and Banno with permission from Fragrance Journal Ltd.[42]

dehydration,[39–41] whereas no similar report is found for trehalose. The rate of hydrolysis has a profound effect on the stability of labile biologicals, as reducing monosaccharides, such as glucose, can undergo Maillard reaction (or browning reaction) readily.

Furthermore, in applications such as in tablet formation, crystalline trehalose may have an advantage over other sugars in that it does not absorb water readily (Fig. 3),[42] thus resulting in tablets of reduced stickiness and enhanced stability.

Many of the differences observed in the physical properties between trehalose and sucrose may arise from their variance in flexibility or conformation. In most of the common disaccharides, the conformation is influenced by intramolecular hydrogen bonds present between the monosaccharide residues. For trehalose in the crystalline form, no direct intramolecular hydrogen bonds have been reported[50] such as those found in sucrose.[51] However, the presence of indirect inter-residue hydrogen bonds, through water, has been reported;[50,52] the average bond length is

Table 1. Comparison of Properties of Two Disaccharides: Trehalose and Sucrose

Properties	Trehalose	Sucrose	References
Solubility (g/100 g H_2O, at 20°C)	40.6–68.9[a]	200	27,33
Melting temperature (°C)	210–215[b]	188	27–30
Glass transition temperature (T_g, °C)	110–120	65 –75	28–29,34
Relative viscosity[c]	1.85	1.3	42–43
# Equatorial –OH	8	6–7	44
Diffusion coefficient (cm^2/s)[d]	1.91×10^{-8}	5.89×10^{-8}	45
Density (g/cm^3, at 25°C and 85°C)	1.58, 1.41	1.59, 1.37	46–47
Hydration number[e]	11	8	46
Rate of hydrolysis (s^{-1}, at 25°C)[f]	3.3×10^{-15}	5.0×10^{-11}	38
Stability in extreme pH (% remaining)[g]	>99%	~0% at pH 3–4	27,42
Acrylamide formation[h]	0 mg/mol Asn	98 mg/mol Asn	27
Calcium dissolution in phosphate buffer[i]	24 ppm	6 ppm	27,48–49
Sweetness[j]	45%	100%	27

[a] Wide range of solubility is due to the difference in purity of trehalose used in the studies.
[b] Melting temperature of anhydrate trehalose crystals.
[c] Viscosity of sugar solutions (0.5 M) with respect to water (viscosity = 1) at 25°C, as measured by Cannon-Manning semi-Micro-type capillary viscometer.
[d] Diffusion coefficient of disaccharides (74 wt %) was measured using pulsed-gradient-spin-echo NMR at 50°C.
[e] Hydration number is defined as the average number of water molecules that are hydrogen bonded to the sugar molecule, as computed by molecular simulation. The hydration numbers for sucrose and trehalose were computed for 50 wt % sugar solutions at 80°C and 87°C, respectively.
[f] Hydrolysis rate constants of disaccharides (0.05 M) in potassium phosphate buffer (0.1 M, pH 8.1) were determined using protein NMR at high temperatures and extrapolated to obtain the values at 25°C.
[g] In a pH range from 3.5 to 10 at 100°C for 24 h.
[h] Amount of acrylamide formation in a mixture containing asparagine and disaccharide, both at 0.1 mmol concentration, was measured by GC. The solution was heated at 150°C for 20 min.
[i] In a 50 mM phosphate buffer at pH 6.8 and 10% solution of either disaccharide.
[j] Sweetness with respect to that of sucrose.

relatively short (1.825 Å), thus the interaction is unusually strong.[53] The angle at the ring oxygen atoms has been reported to be 114.1° and 116.1° for trehalose and sucrose, respectively, whereas the angle at the glycosidic oxygen is reported as 115.8° and 114.4° for the two sugars.[50,54] Other crystallographic data for the two sugars can be found elsewhere.[50,54–58]

Trehalose is also differentiated from the other disaccharides by its high number of equatorial — OH groups,[44] resulting in stronger interactions with water in solution and the relative ease with which it can include itself in the water cluster. Sucrose, on the contrary, does not integrate itself well in the water cluster, creating a larger structure in comparison with that for trehalose.[44] In fact, sucrose is consistently less hydrated than trehalose (Fig. 4). The practical implication for this difference is that trehalose-containing formulations may be more difficult to dehydrate to low levels of residual water content (i.e., <1%) than the corresponding sucrose-based formulations.

In addition to the reasons given above, this difference may be attributed to the number of intramolecular hydrogen bonds found in the two disaccharides (particularly, at high concentrations); trehalose has been reported to form only one such bond in comparison with two for sucrose, thus there are more sites available for trehalose to hydrogen bond with water, resulting in higher hydration number.[46,59] With increasing trehalose concentration (from 5 to 90 wt %), the hydration number was shown to decrease monotonically, from approximately 13 to 3 (Fig. 4).[46] This

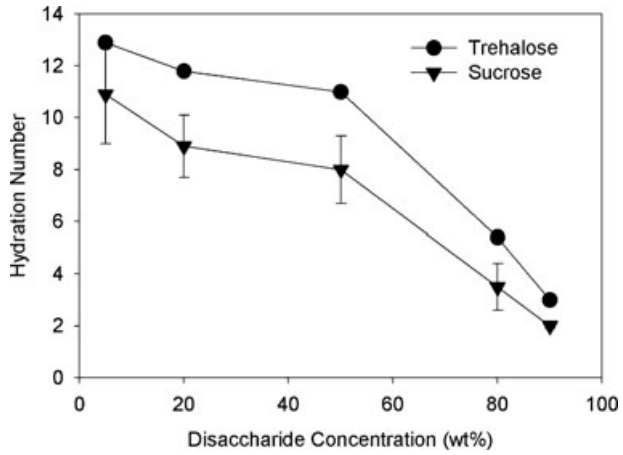

Figure 4. Hydration number of trehalose and sucrose as a function of sugar concentration (wt %). The data for trehalose and sucrose were calculated at 87°C and 80°C, respectively. The hydration number is defined as the average number of water molecules that are hydrogen bonded to the sugar molecule, with the following criteria: oxygen–oxygen distance of <3.4 Å and H—O—O angle that is >120°. Reproduced from Ekdawi-Sever et al.[46] with permission from ACS Publication.

reduction is due to the scarcity of water in the vicinity of the sugar molecules, forcing them to satisfy the hydrogen bonding requirements by creating intramolecular hydrogen bonds. This arrangement results in a folded configuration, and thus the reduction in hydration number.[45–47] Fourier transform infrared (FTIR)-attenuated total reflectance (ATR) and laser-Raman spectroscopic techniques were used to demonstrate the folding of sucrose molecule around its glycosidic bond (with increasing concentration), whereas no such configurational changes were observed for trehalose.[60] The effect of conformational differences between the two sugars was also noted in their varying degrees of interaction with calcium ion[48] (Table 1). As will be discussed next, the stronger and more prominent hydrogen bond network for trehalose, compared with that for sucrose, has an impact on several solution properties, namely diffusion and viscosity.

Comparison of trehalose with sucrose revealed that the former demonstrated lower water diffusion coefficient and higher viscosity, although the density was similar.[46] All three properties are interconnected. It has been suggested that with increase in density, the free volume decreases, which, in turn, causes a decrease in diffusivity and thus increase in viscosity.[61] The diffusion coefficient for sucrose was shown to be higher than that for trehalose, particularly at high concentrations; at 74 wt % sugar, the diffusion coefficients were determined to be 5.89×10^{-8} and 1.91×10^{-8} cm^2/s for sucrose and trehalose, respectively.[45] The faster diffusion coefficient for sucrose can be attributed to its smaller hydration number in comparison with trehalose (Fig. 4); as the hydrated sucrose is smaller in size compared with trehalose, it can diffuse more readily. At lower concentrations, however, there is significantly more "bulk" water in the system that is insensitive to the identity of the disaccharide involved, thus no significant difference in diffusion is observed. With enhanced restriction in mobility of the sugar system, the viscosity is expected to increase. In fact, Sola-Penna and Meyer-Fernandes[43] have reported the viscosity of trehalose to be greater than that for sucrose, and the difference was shown to increase at higher sugar concentrations (Fig. 5).[43] This suggests that in applications that require high concentrations of proteins, formulation with trehalose will result in even higher overall viscosity than that prepared with sucrose, which is certain to have an impact on the delivery system being evaluated.

The unique properties of trehalose discussed above may be further enhanced through the incorporation of secondary components, such as salts and other saccharides. For example, the addition of cations and borate has been shown to increase the stability of various enzymes formulated in trehalose[62,63] whereas phosphate was demonstrated to improve the stability

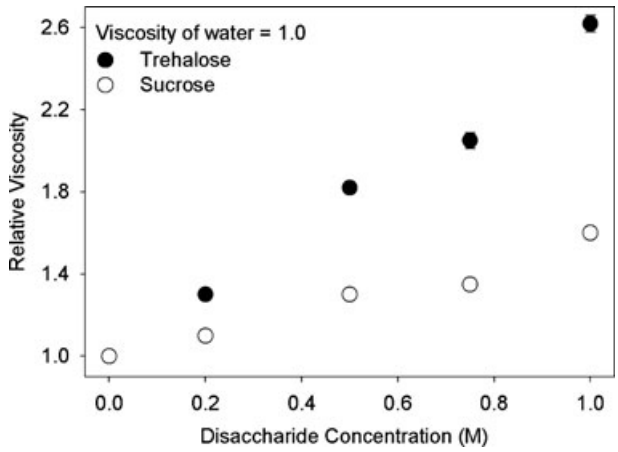

Figure 5. Relative viscosity of trehalose and sucrose solutions at concentrations ranging from 0 to 1 M. The viscosity was measured using a Cannon-Manning semi-Micro-type capillary viscometer and water was used as standard (viscosity = 1.0). Reproduced from Sola-Penna and Meyer-Fernandes.[43] with permission from Academic Press.

of liposomes[64] and platelets.[65] In all of these applications, the ionic species are thought to increase the strength of the interactions present within the glassy matrix, through enhanced hydrogen bonding network, and demonstrated increased T_g. In another application, the addition of glucose at 1:10 weight ratio improved the storage stability of glucose-6-phosphate dehydrogenase[66]; glucose incorporation was thought to lower the free volume by filling in the void in the trehalose–protein glass, thus reducing molecular mobility, and consequently, improved stability.

Mechanism of Stabilization

Trehalose has been utilized to stabilize simple systems, such as lipids and proteins, as well as more complex biologicals, including viruses, bacteria, and tissues. The unique physical properties of trehalose, which are responsible for its stabilizing effects, have been discussed in the previous section. Here, two mechanisms will be described that explain these effects. The first mechanism involves "direct interaction" of trehalose with the compound and encompasses stabilization in the dry state, through hydrogen bonding with proteins, as well as in solution, through interaction with metal ions to suppress the degradation of vitamins. The second mechanism involves the effect of trehalose on the environment surrounding the compound ("indirect interaction") through restriction of mobility and increased hydration.

Direct Interaction

One of the reasons why labile biologicals, such as proteins, lose activity during freezing and/or drying is the change in conformation caused by the removal of water. During dehydration, the various interaction forces responsible for maintaining the protein structure are disrupted and results in structural changes (i.e., denaturation). Polyhydroxy compounds, such as trehalose and sucrose, have the ability to suppress these changes by hydrogen bonding with the protein surface, resulting in the maintenance of protein conformation and thus activity (water replacement hypothesis). All biological macromolecules are typically stabilized by water that forms hydrogen bonds around the molecules. The direct interaction between trehalose and lysozyme, upon freeze drying, was demonstrated using FTIR;[67,68] in the absence of trehalose, the amide II band of lysozyme broadened and shifted from 1543 to 1530 cm^{-1}, whereas in its presence (100 mg/mL), the amide II band was maintained at 1542 cm^{-1}.[67] This suggests that lysozyme freeze dried in the presence of trehalose demonstrated similar spectrum to that observed in the hydrated state. Alternatively, the FTIR spectrum of freeze dried trehalose in the presence of lysozyme was shown to be similar to that of hydrated trehalose. Furthermore, both spectra were qualitatively different from that of freeze dried trehalose (alone) or crystalline trehalose. These observations suggest that trehalose can replace water in the hydrogen bonding network of lysozyme upon freeze drying, and similarly, lysozyme can substitute for water in hydrogen bonding with trehalose; that is, there is a direct interaction between trehalose and lysozyme. In the case of *Humicola lanuginosa* lipase (HLL) freeze dried with trehalose (300 mM), stabilization of enzyme structure was found to correlate with maintenance of activity upon reconstitution.[69] The strong interaction present between trehalose and protein surface may be due to the conformation or flexibility of the disaccharide, as described previously, resulting in enhanced interaction with the irregular surface (polar groups) of macromolecules.[25] However, other properties besides conformational flexibility may be responsible for the observed differences in protein stabilization, as sucrose incorporation has been reported to improve the stability more so than trehalose in some reports.[70,71] Thus, the superiority of trehalose as a stabilizer is not universal. It is evident, however, that direct interaction to the protein surface is insufficient for stabilization, as evidenced by the lack of stability conferred to phosphofructokinase (PFK) by glucose and other monosaccharides.[72]

Trehalose has been reported to interact with metal ions such as Ca, Fe, and Cu. In the case of calcium ions, trehalose addition inhibited their precipitation from phosphate buffer (pH 6.8) in a dose-dependent manner, which is thought to occur through its direct interaction with calcium, thereby inhibiting the formation of insoluble calcium phosphate.[48] The interaction of trehalose with calcium has been verified

using ^{13}C nuclear magnetic resonance (NMR) spectroscopy.[48] Furthermore, at 10% addition, trehalose was able to increase the amount of soluble calcium in a mixture of $CaCl_2$ in 50 mM K-$NaPO_4$ to approximately 24 ppm, whereas in sucrose, approximately 6 ppm soluble Ca was observed. In another application, trehalose was observed to suppress the elution of Mg ions from pork meat and spinach.[27] The affinity of trehalose for other metal ions, such as iron and copper, has also been reported and demonstrated to result in stabilization of vitamin C,[48] as will be described in more detail later.

Besides proteins and ions, trehalose has also been reported to interact with unsaturated fatty acids such as linoleic acid. The presence of direct interaction was demonstrated by NMR; the relaxation time of the 9, 10, 12, and 13 positions on olefin of linoleic acid was observed to decrease upon the addition of trehalose, and similarly, the relaxation time of the 3 and 6 positions of trehalose was shown to decrease.[24,73] More specifically, trehalose was found to interact only with the double bond in the *cis*-conformation by OH—π and CH—O types of hydrogen bonding.[73] Furthermore, the degree of relaxation time decrease was dependent on the ratio of trehalose to linoleic acid. These observations suggest the existence of direct interaction of trehalose with linoleic acid, which provides stabilization of fatty acids and results in the suppression of foul odor during heating of meat products. Oku et al.[73] demonstrated by computer modeling that the activation energy of the hydrogen abstraction step (initial step in the oxidation reaction of unsaturated fatty acids) was increased by approximately 10 kcal/mol, which corresponds to a decrease in reaction rate by approximately 10^8. This interaction appears to be specific to trehalose, as sucrose was ineffective in suppressing the formation of volatile aldehydes.[24] The interaction of trehalose with linoleic acid suppressed the formation of hydroperoxide (HPO), which is the initial reaction product of linoleic acid oxidation.[24,74] Stabilization of unsaturated fatty acids using trehalose suppresses oxidation reactions and the formation of free radicals and aldehydes, which are responsible for foul odor. Another point to note from these observations is the ability of trehalose to interact with nonpolar groups. The mechanism of trehalose interaction with phospholipids is well established and will be discussed in greater detail in the section entitled *Research Applications*.

The foul odor produced during heating of milk is the result of dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) formation, caused by the degradation of cystine and methionine residues. The addition of trehalose was reported to suppress the formation of foul odor and reduced the amount of DMS and DMDS formed to 66% and 100% of the control, respecitvely.[27] This is thought to occur through the direct interaction of trehalose with the amino acid groups, thus suppressing the formation of sulfur compounds. Sucrose, on the contrary, was demonstrated to be not only ineffective, but rather, it enhanced the formation of these sulfur-containing compounds. The foul odor of fish produced during boiling was also reduced in the presence of trehalose and was attributed to the suppression of trimethylamine (TMA) formation from trimethylamine-N-oxide (TMAO).[75,76] The stabilization was demonstrated to occur through hydrogen bonding by NMR and computer simulation analysis;[77] the 2, 3, and 2' positions of trehalose were shown to hydrogen bond with TMAO. Furthermore, DSC analysis of trehalose–TMAO mixture revealed the disappearance of pure trehalose and TMAO peaks and the formation of a new peak with diminished enthalpy at a lower temperature.[77] Trehalose was also demonstrated to interact with other saccharides such as glucose. In one application, the formation of acrylamides was shown to decrease in the presence of trehalose. Acrylamides are produced through the interaction of asparagine (Asn) residues and reducing sugars during heating and processing of food products such as potato chips and cereal. The addition of trehalose was shown to suppress the formation of acrylamide through its interaction with glucose, which, in turn, suppressed the glucose–Asn reaction.[27] These effects will be described in more detail later.

Indirect Interaction

Owing to its unique physical properties, as described above, trehalose is able to impart several "indirect" effects on labile biologicals. The addition of trehalose to a protein-containing solution has been reported to result in its exclusion from the vicinity of the protein.[78] This results in high amount of water molecules near the protein surface, and suppresses the protein's propensity to denature, which otherwise would have exposed the hydrophobic residues to the polar medium. Thus, trehalose addition results in increased protein stability, as evidenced by the increased denaturation temperature (T_m);[79,80] for example, in the presence of 1 M trehalose, the T_m of RNase A increased from 40.9°C to 50.9°C.[80] As a consequence of increased protein stability, its propensity for unfolding[78,81,82] and/or aggregation[83,84] is also inhibited. Furthermore, the increase in the T_m, upon the addition of trehalose, has been reported to correlate to the increase in the surface tension at the protein–water interface;[79,80] the addition of 1 M trehalose resulted in T_m increase by 10°C and surface tension increase by 1.69 dyne/cm.[80] For a more detailed explanation, the reader is referred to the work of Timasheff and coworkers.[78,80,85–88] The presence of trehalose, however, is not always beneficial, as demonstrated by Singer and Lindquist[83] in their study of yeast cells recovering from heat shock; although trehalose was able

to stabilize the denatured proteins (luciferase) in the non-native state *in vivo*, its extended presence and high concentration inhibited the reactivation of denatured proteins, thus necessitating its removal during recovery from heat shock.

Sugars, such as trehalose, can form an amorphous matrix upon dehydration, serving to restrict the mobility of biomolecules.[35,66,70] This retards the rate of chemical degradation and maintains the separation between the neighboring biomolecules, which, in the absence of sugars, may have aggregated.[70,89] Trehalose is unique in that it forms a glassy structure that is stable at high temperatures,[90] which allows its use in a wide range of extreme environmental conditions. The glassy state maintains the entrapped biomolecules in a form that allows their return to native structure and function following rehydration.[25,90,91] This property of trehalose is essential for the long-term stability of chemically unstable proteins. In case of lipidic membranes, their encapsulation within the glassy matrix has been reported to result in inhibition of fusion[92,93] maintenance of lipid phase distribution,[94] as well as in avoidance of lipid phase transitions,[34] all of which contribute to membrane stabilization.[72] Vitrification alone, however, is insufficient to confer stability. Koster et al.[36] have reported that steric effects play a significant role in the ability of saccharides to stabilize lipid membranes. In one study, several saccharides and polymers, varying in their dry T_g values as well as in their molecular size, were incorporated into a model cell membrane composed of phosphatidylcholines, and subsequently dried. Interestingly, excipients with high T_g values, such as dextran and polyvinylpyrrolidone, were not always effective in stabilizing the lipid membrane. Koster et al.[36] concluded that those excipients were ineffective in stabilizing the lipid membranes because they are excluded from the inter-bilayer space during dehydration through steric hindrance and phase separation. Crowe et al.[34] demonstrated the lack of direct correlation between the T_g of various saccharides and the gel-to-liquid crystalline phase transition temperature (T_m) of dipalmitoylphosphatidylcholine (DPPC). Rather, the effect of sugars on the phosphate asymmetric stretch of the phospholipid head groups was shown to give a better prediction (i.e., direct interaction). This suggests that indirect interaction alone may not be sufficient to explain the observed effects. In another example, the fragility of sucrose and trehalose glasses was compared by examining the correlation between the relaxation time constant (τ) and storage temperature through the use of Vogel–Tammann–Fulcher equation.[70] Although amorphous sucrose has a lower T_g in comparison with trehalose at identical residual water contents ($\sim$60°C compared with $\sim$110°C),[28,95,96] due to its higher fragility, τ for sucrose exhibits greater nonlinearity. This in turn can result in a matrix with lower molecular mobility at temperatures below T_g, which may lead to improved stability. Thus, the selection of stabilizers based solely on their T_g values can be misleading and may not provide sufficient stability to lipid membranes and other lipid-containing biological compounds such as cells, bacteria, and enveloped viruses. In addition to the higher T_g, trehalose possesses greater conformational flexibility in comparison with sucrose, as described previously, which may explain its efficacy in several systems.[97,98]

The initial step of the freeze drying process involves the freezing of the solution; more specifically, the phase separation of water as ice and concentration of the remaining solution. The concentration continues to increase (even beyond the solubility limit, depending on the rate of temperature decrease) until the viscosity becomes sufficiently high to suppress molecular mobility and reaction. Just as the dry state is characterized by the T_g, the frozen concentrate is characterized by T_g', the glass transition temperature of maximally concentrated solution; below T_g', the mobility is suppressed by the glassy matrix, whereas above T_g' (in the rubbery state), the molecules have sufficient mobility, allowing for reaction to occur. The T_g' of trehalose has been reported to be in the range of -22°C to -40°C, and this wide variance is due to differences in measurement methodologies that were employed.[33,96] As the T_g' of trehalose is higher than that of sucrose (-40°C in comparison with -46°C, measured using the same technique),[96] it is possible to conduct primary drying at a higher temperature for trehlaose-containing formulations, which reduces the load on the lyophilizer and minimizes the effect of cold denaturation. Thus, in the frozen state, as was described in the dried state, the suppression of molecular mobility and separation of reactant molecules by the glass-forming excipients, such as trehalose, result in improved storage stability of labile biologicals. Another aspect to keep in consideration is the distribution of excipients during the freezing process. Stabilizing excipients in solution, as described previously, are preferentially excluded from the surface of proteins. Carpenter and Crowe[99] demonstrated that similar mechanism is responsible for improving the stability of a model protein, lactate dehydrogenase, to freezing stress, which corroborates the earlier findings of Franks.[100]

There are complications to the use of trehalose. These issues are primarily concerned with the crystallization of trehalose during freezing and/or lyophilization. As mentioned previously, trehalose can adopt several polymorphic configurations in the crystalline phase, depending on the water content and processing method (i.e., rate of dehydration, heating, etc.).[29] For many years, trehalose has typically been reported to

be in the amorphous phase following lyophilization, spray drying, etc. Although, this may be true about the final product, it does not preclude the possibility of trehalose crystallization during processing and its subsequent conversion to the amorphous state, prior to reaching the final dried product. In fact, trehalose crystallization, initially as dihydrate and subsequently as anhydrate, was observed in the frozen solution during annealing. The crystals were converted to amorphous anhydrate following secondary drying.[101] Thus, analyzing the final dried product alone may not be sufficient to reveal the full extent of the crystallization behavior of trehalose during processing. Crystallization can be deleterious to the stability of labile biologicals, as it can disrupt the network of hydrogen bonds present in the amorphous carbohydrate matrix. The authors do note, however, that annealing was necessary to induce crystallization, thus its presence and extent is unclear in the absence of annealing. One notable exception was reported by Carpenter and Crowe[67] for a system consisting of lyosozyme and trehalose. Furthermore, the occurrence of trehalose crystallization upon scaling-up the lyophilization process remains unknown. In addition to the traditional techniques to assess the crystallinity of a sample, including X-ray diffraction, DSC, and NMR spectroscopy, novel methods with increased sensitivity to crystallinity, in the presence of ice, are being developed, including near infrared and Raman spectroscopy.[102] It should be noted that the occurrence of crystallization in the solid dosage form of amorphous small molecule may not be deleterious.

Trehalose can also be included to modify the osmolarity of the medium, in particular for microorganisms to increase their resistance to osmotic stress and desiccation (osmoadaptation). For example, the inclusion of 10 mM trehalose in the growth media of *Aspergillus parasiticus* resulted in increased resistance to high osmolarity (27.5% NaCl).[103]

Table 2 shows a list of inventions that utilize the unique properties of trehalose in a variety of applications, including tablet formation, medical applications, and food stabilization. The broad general categories, as shown in the table, will be discussed in more detail later.

NATURAL OCCURRENCE

Close to 100 species representing plants, algae, fungi, yeasts, bacteria, insects, and other invertebrates are listed by Elbein[26] to contain trehalose. Trehalose has been isolated from various seed plants, including sunflowers,[26] and is found in yeast and fungi, in which the common occurrence is in spores, fruiting bodies, and vegetative cells.[104,105] Trehalose is also present in high concentrations in Baker's and Brewer's yeast[106,107] as well as in insects.[108,109] More specifically, trehalose was isolated from several species of insects and was the principal sugar (80–90%) found in the hemolymph. Furthermore, it can comprise approximately 20% of all carbohydrates during specific stages of insect development.[108]

Just as the species that contain trehalose are diverse, the role of trehalose in each of these species varies widely. The most prevalent and widely known effect is its ability to enhance desiccation tolerance. Resurrection plants *Craterostigma plantagineum* and *Selaginella lepidophylla*, which contain more than 10% dry weight of trehalose, have been demonstrated to withstand heating to 100°C without damage in the fully dried state and resume activity upon rehydration, even following prolonged storage at room temperature.[110,111] Similarly, certain mushrooms that contain trehalose (up to 20%) have been found to be stable on storage at room temperature for several years (in the dry state), and promptly rehydrate to produce a fresh product.[110] The high trehalose content in Baker's yeast allows for its prolonged survival on the kitchen shelf as a dry product, which upon rehydration, immediately begins the metabolic production of CO_2.[112,113] Brine shrimp embryos entering dormancy accumulate trehalose approximately up to 10% of their dry weight, and trehalose is thought to act as both a stabilizer against desiccation and an energy source for the embryos emerging from dormancy.[114] In another application, the concentration of trehalose was associated with nematode survival during desiccation.[18,115] Furthermore, cultures of yeast have shown accumulation of trehalose with growth; low concentrations during log phase and higher concentrations in the stationary phase, which coincided with enhanced desiccation tolerance.[116]

Trehalose has also been demonstrated to provide cold protection in several organisms.[18] For example, the prepupal larvae of the sawfly *Trichiocampus populi*, which contain high concentrations of trehalose, were found to survive at −30°C for several hours.[117] In lower organisms, trehalose acts as an energy source during certain stages of development, such as early germination of spores.[26,118] In mycobacteria and corynebacteria, trehalose can be incorporated into the glycolipids, as a structural component,[26,119] whereas in other microorganisms, derivatives of trehalose can act as either metabolic intermediate or structural component;[26] for example, trehalose-dimycolate, which serves as a cell wall lipid of *Mycobacterium tuberculosis*.[120] Trehalose is also an important component in fungal spores, in which trehalose hydrolysis plays a major role during early germination, and thus serves as a source of carbon for synthesis and glucose for energy.[121,122]

There are several mechanisms by which trehalose synthesis occurs. Nutrient limitation is a common

Table 2. List of Inventions Utilizing Trehalose; US Filing Only, and Arranged by Filing Dates

Patent # (Filing Date)	Assignee	Description
Formulation		
6,991,790 (Nov 2000)	Genentech	Antibody formulation containing 2%–10% (w/v) trehalose
6,821,515 (Aug 2000)	Genentech	Lyophilized antibody formulation containing trehalose at 100–600:1 mol ratio of trehalose:antibody
5,804,557 (Sep 1996)	Genentech	Method of increasing polypeptide solubility with trehalose addition (1:2–1:20 trehalose:polypeptide)
Stabilization		
11/340,483 (Jan 2006)	Canadian Blood Services	Delivery of trehalose (0.05–0.5 M) using liposomes to cells to enhance cryostability
10/993,468 (Nov 2004)	Wisconsin Alumni Research Foundation	The use of trehalose to cryopreserve pluripotent stem cells on solid support matrix
7,314,755 (Oct 2004)	Board of Supervisors of Louisiana State University and A & M College	Extracellular agent (i.e., ATP) used to create pores on the membrane of vertebrate cells to incorporate trehalose, which is then effectively desiccated
7,169,606 (Mar 2004)	Wisconsin Alumni Research Foundation	An aqueous medium for preserving platelets containing trehalose at 10–300 mM
7,270,946 (Sep 2003)	Organ Recovery Systems, Inc.	A method for preserving living mammalian cellular material by incubation in trehalose at 0.2–0.4 M followed by desiccation
10/108,344 (Mar 2002)	Naval Medical Research Center	Preservation of bacteria grown in 10–200 mM trehalose dried at room temperature
6,770,478 (Jan 2002)	The Regents of the University of California	A process of loading trehalose into an erythrocytes, requiring removal of cholesterol and incubation near the phase transition temperature
6,723,497 (Apr 2001)	The Regents of the University of California	A method for preparing dry platelets by freeze-drying, following incubation in trehalose solution ($\leq$50 mM) at a temperature near the phase transition temperature
6,528,309 (Mar 2001)	The Regents of the University of California	Method for desiccation of mammalian cells using trehalose
6,475,716 (Mar 2001)	Biobank, Co., Ltd.	A method for preservation of mammalian organs in trehalose solution
6,653,062 (Jul 2000)	Wisconsin Alumni Research Foundation	Description of preservation medium containing 5–60 wt % trehalose and phosphate ions
6,610,531 (Sep 1998)	US Navy	Preservation of aerobic bacteria dried with 10–200 mM trehalose and divalent cation
6,221,575 (Feb 1998)	Quadrant Holdings Cambridge Ltd.	A method for preserving platelets with 5–150 mM intracellular and 1%–30% extracellular trehalose
6,005,100 (Jun 1997)	Hayashibara Biochemical Laboratories	Composition for prolonging the shelf life of food, pharmaceutical, and cosmetic products containing trehalose and one of lactic acid, citric acid, or ethanol
5,827,741 (Nov 1996)	The Regents of the University of California	Method for incorporating 10–1500 mM trehalose into eukaryotic cells during phase transition of lipid bilayers
5,242,792 (Feb 1991)	US Navy	Method for preservation of mammalian red blood cells by freeze-drying with 300–500 mM trehalose
4,806,343 (Mar 1986)	University of Southwestern Louisiana	A method of stabilizing artificial red blood cells by freezing with trehalose and a transition metal ion

(*Continued*)

Table 2. Continued

Patent # (Filing Date)	Assignee	Description
Delivery vehicle		
6,187,330 (Jan 1999)	Scios Inc.	Composition for the controlled release of a peptide or protein encased within trehalose glass
5,962,310 (Jul 1998)	Becton Dickinson and Company	Use of trehalose as a vehicle for delivery of particles to cells during agitation or sonication
Pharmaceutical Applications: Inhibition of Radical Reaction		
10/525,839 (Aug 2003)	Hayashibara Biochemical Laboratories	A composition for inhibition of radical reaction consisting of cyclotetrasaccharide and trehalose
Pharmaceutical Applications: Solid Dosage Application		
7,575,762 (May 2007)	Astellas Pharma	A method for manufacturing sustained-release fine particles embedded within a quick-disintegrating tablet, in which trehalose serves as a binder
7,425,341 (Sep 2007)	KV Pharmaceutical Company	Composition for rapidly disintegrating tablet, including trehalose, in which the tablet has a friability of $\leq$1.5% and porosity from 15% to 45%
7,074,428 (Sep 2003)	Astellas Pharma	Composition for a quick-disintegrating tablet in buccal cavity, in which trehalose is used to spray coat the tablet and/or as a binder
6,998,139 (Mar 2002)	Astellas Pharma	A method for preparation of dry-coated tablet, in which the bitter-tasting active agent is coated with trehalose
6,589,554 (Sep 2000)	Yamanouchi Pharmaceutical Co., Ltd.	A composition for preparing a quick-disintegrating tablet in buccal cavity, using trehalose, resulting in tablet hardness of about 3.1 kp or more
6,455,053 (May 2000)	SSP Co., Ltd.	A method for producing an uncompressed, rapidly dissolving solid preparation with 10–30 wt % trehalose
6,740,339 (Dec 2001)	Takeda Chemical Industries, Ltd.	A composition for preparing a quick-disintegrating solid preparation comprising trehalose and a cellulose compound
6,194,001 (Jun 1999)	Quadrant Holdings Cambridge Ltd.	Tablet composition consisting of clavulanic acid, amoxicillin, and trehalose (5%–50%, amorphous form)
5,958,455 (Feb 1996)	Quadrant Holdings Cambridge Ltd.	Tablet composition comprising an anhydrous trehalose serving as a diluents and an active agent
5,762,961 (Feb 1996)	Quadrant Holdings Cambridge Ltd.	A method of preparing rapidly soluble tablets composed of active agent, diluent, binder, and volatile salts, in which trehalose is used as a diluents
6,290,991 (Dec 1994)	Quadrant Holdings Cambridge Ltd.	Preparation of powder suitable for administration by inhalation, wherein the powder comprises trehalose and a bioactive agent
4,678,812 (May 1986)	E.I. Du Pont De Nemours and Co.	Method of tableting powders produced by S-1 spray freezing process using trehalose as tableting excipient and stabilizer

(*Continued*)

Table 2. Continued

Medical Applications	Patent # (Filing Date)
Antiosteoporosis	10/513,119 (May 2002), 6,440,446 (Apr 1999)
Articular failure	7,214,667 (May 2005), 5,981,498 (Nov 1998)
Neurofunction	6,232,294 (Dec 1998)
Osteoclast formation	10/492,703 (Oct 2002)
Ophthalmic use	7,732,425 (Mar 2003)
Mucasal immonomodulator	10/765,905 (Jan 2004), 10/169,670 (Jul 2002)
Food Applications	Patent # (Filing Date)
Suppression of foul odor	11/578,336 (Apr 2005), 10/540,772 (Dec 2003), 6,576,281 (Dec 1998)
Flavor modification	11/061,015 (Feb 2005), 6,432,470 (Apr 1998), 6,159,529 (Jun 1997)
Preservation	10/920,205 (Aug 2004), 6,641,853 (Jan 2000), 6,254,912 (Apr 1999)
Quality improvement	10/835,920 (Apr 2004), 10/536,268 (Nov 2003), 10/535,255 (Nov 2003), 7,186,824 (Sep 2003)
Mineral absorption	10/565,069 (Jul 2004)
Nutritional supplement	6,596,702 (Jun 2002)
Food Additive/ingredient	6,620,791 (Apr 2002), 6,455,096 (Apr 1999)
Cosmetic Applications	Patent # (Filing Date)
Volatile aldehyde suppression	10/545,078 (Feb 2004), 6,497,862 (Mar 2001), 6,268,353 (Sep 1999)
Skin application	10/545,166 (Feb 2004), 10/477, 147 (Nov 2003)

cause of trehalose accumulation in yeast and other fungi,[121] whereas in other organisms, increase in the levels of substrate for trehalose synthase (TS) leads to increased levels of trehalose.[123] In Brewer's yeast and in several other organisms, trehalose synthesis is catalyzed by enzymes that facilitate the reaction of UDP-D-glucose with D-glucose-6-phosphate,[26,124] as will be described in more detail later. Interestingly, upon the removal of stresses that caused the upregulation of trehalose synthesis, the synthesis is downregulated and the sugar is rapidly broken down. In the case of yeast, the resumption of growth through the addition of a carbon source (or any other nutrient that was initially deficient) resulted in the rapid breakdown of accumulated trehalose,[121] whereas for cells exposed to heat shock, the trehalose level rapidly declined upon transferring the cells back to lower temperatures.[123] As the accumulation of trehalose may cause problems with osmolarity, inhibition of key metabolic reactions, or be toxic in other ways, regulation of trehalose levels in the above-described systems is critical.

Although there appear to be many organisms from which trehalose isolation is possible, trehalose was considered a rare sugar because it could only be isolated in relatively large amounts from resurrection plants (~1.5 wt %) and trehala manna (20–25 wt %), and the purification procedure was rather tenuous.[125] Over the years, synthesis procedures were developed that aimed for large-scale production of trehalose with high yield and purity, which were hoped to lead to the availability of trehalose at an affordable cost and its widespread use. These methods utilized organisms that are more readily available,[7] as will be described in the next section.

METHOD OF PRODUCTION

Trehalose production has progressed over the years from gram scale to tons through the development of novel synthesis techniques. In nature, trehalose is synthesized by a variety of organisms using a combination of enzymes and starting material. In Brewer's yeast, trehalose synthesis is carried out by enzymes that catalyze the reaction of UDP-glucose and glucose-6-phosphate to produce trehalose-6-phosphate, which is then dephosphorylated.[124] Examples of organisms that are known to synthesize trehalose by this mechanism include yeast, bacteria,[26,30,124,126] insects,[127,128] *M. tuberculosis*,[129] and *Dictyostelium discoideum*[130] to name a few. These enzymes have been isolated, purified, and used for bench-scale production of trehalose. In *Pimelobacter* sp., TS was shown to catalyze an intramolecular rearrangement of maltose to convert the α-1,4- to α-1,1-glycosidic linkage of trehalose.[131] In another example, trehalose synthesis in bacteria was found to utilize maltooligosaccharide or starch as the starting material: maltooligosyltrehalose synthase catalyzes the conversion of maltodextrin to maltooligosyltrehalose and then maltooligosyltrehalose trehalohydrolase hydrolyzes this product to form trehalose.[132] Other techniques developed for trehalose synthesis include chemical synthesis, microbial fermentation, enzymatic conversion of maltose, and transgenic technology;[30,107,124–126,133–138] however, none of these methods were successfully implemented for large-scale production. In all cases, the main reason appears to be the cost (i.e., scalability). For this reason, viable commercialization and widespread use of

trehalose were restrained, except in cases of high-premium cost products, i.e., pharmaceuticals.

Recently, Hayashibara Biochemical Laboratories (Okayama, Japan) reported a novel synthesis method, which dramatically reduced the cost of trehalose production.[139] The starting material for trehalose synthesis is starch derived from either corn or tapioca, both of which are low cost. Although the synthesis utilizes a mixture of several enzymes, the two main enzymes that directly produce trehalose from starch are maltooligosyl-trehalose synthase (MTSase) and maltooligosyl-trehalose trehalohydrolase (MTHase).[140–142] These two enzymes were derived from a nonpathogenic soil microbe designated as *Arthrobacter ramosus*.[31,143] The key function of MTSase is to recognize the reducing end of the terminal D-glucose units of amylose molecules[141–142] and to convert the α-1,4- to α-1,1-glycosidic bond in an intramolecular transglycosylation reaction, creating an amylose molecule with a terminal trehalose unit. MTHase then hydrolyzes the α-1,4-glycosidic bond between the second and third D-glucose units, resulting in the detachment of trehalose from the end of the amylose chain.[142] These two enzymes can repeatedly act on α-1,4-glucan to produce trehalose, typically resulting in yield no greater than 80%. However, the efficiency of trehalose production is limited by the degree of branching present in starch, as the enzymes can only act on the linear segments. The amount of branching can be reduced by the addition of isoamylase, which further enhances process efficiency.[31] As a side product during trehalose synthesis, several low-molecular-weight dextrins, such as glucose, maltose, and maltotriose, are produced. To increase the process yield even further, cyclomaltodextrin glycanotransferase is used to convert these low-molecular-weight dextrins into high-molecular-weight compounds, which can be converted and broken down by MTSase and MTHase to trehalose.[31,139] Prior to the action of these two enzymes, there are several processes that take place on the starting material. Briefly, the process consists of (1) suspension of starch to produce a slurry, (2) heating and liquification of slurry by the addition of α-amylase, (3) inactivation of α-amylase by heating, (4) cooling of the slurry, and (5) further breakdown of starch and oligosaccharides with enzymes, including isoamylase, cyclodextrin glucanotransferase, α-amylase, and glucoamylase.[7,31] Following the production of trehalose by MTSase and MTHase, several purification processes are conducted, including (1) decolorization of solution with activated carbon; (2) filtration of insoluble substances and carbon; (3) ion exchange to remove salts and proteins; (4) concentration of suspension by evaporation; (5) crystallization of trehalose; and (6) recovery of crystals by centrifugation, wash, drying, and granulation.[7] The process results in a dihydrate crystal of greater than 98% purity. Approximately 30,000 ton/year of trehalose is being produced, utilizing this synthesis procedure, and can be found in over 8,000 products encompassing the food, cosmetic, and pharmaceutical fields.[27]

Another consideration in the purification process is the intended use of trehalose. Purity of trehalose for food application can be lower than that required for cosmetics, and even lower than that intended for pharmaceutical products. For this reason, the cost of trehalose will vary depending on the application. This should lower the barrier for the use of trehalose in both the food and nutraceutical industries as well as in every-day use products, as high purity (and high cost) trehalose is not necessary. Varying levels of purity, however, indicates the presence of unwanted side products. These can be critical, depending on the intended application of trehalose, and may include arsenic, heavy metals, microbes, reducing sugars, and endotoxin. Furthermore, cyanide level may need to be monitored if the raw material is produced from a tapioca source. Currently, Hayashibara Biochemical Laboratories is the major producer of trehalose raw material and Ferro Pfanstiehl (Waukegan, IL) and Senn Chemicals AG (Dielsdorf, Switzerland) provide purified trehalose. However, information on their purification procedure is not disclosed, and thus, comparison of their purified trehalose cannot be made.

USE IN BIOTECHNOLOGY AND PHARMACEUTICALS

The use of trehalose in commercialized pharmaceutical products is limited. In the United States, trehalose is only found in four products: monoclonal antibody products Herceptin®, Avastin®, and Lucentis® developed by Genentech (South San Francisco, CA), and a large recombinant protein, Advate®, developed by Baxter (Deerfield, IL) (Table 3). In Japan, there are several other trehalose-containing commercial products, including Sawachion (Sawai Pharmaceuticals, Osaka, Japan), Imidapril (Ohara Pharmaceutical Co., Ltd., Shiga, Japan), and Bio-Three HI (Towa Pharmaceutical Co., Ltd., Osaka, Japan); however, compared with the other saccharides, such as sucrose or mannitol, the number of products is quite few. There are many reasons for the inclusion of trehalose in these formulations, including, but not limited to, high T_g, low rate of hydrolysis, control of osmolarity, and protein stabilization, as described in the previous section.

Although the number of products incorporating trehalose is low, there are several products in development, in which trehalose is being evaluated as one of the formulation components, if not the key component, for product stabilization. For example, in parenteral formulations, trehalose may be a suitable

Table 3. Compositions of Trehalose-Containing Pharmaceuticals Currently on the Market

	Product Name			
	Herceptin®	Avastin®	Lucentis®	Advate®
Company	Genentech/Roche	Genentech/Roche	Genentech/Novartis	Baxter Healthcare Co.
Form	Lyophilized powder	Solution	Solution	Lyophilized powder
Administration	Intravenous	Intravenous	Intravitreal	Intravenous
Active	Trastuzumab, recombinant DNA-derived humanized monoclonal antibody	Bevacizumab, recombinant humanized monoclonal IgG1 antibody	Ranibizumab, recombinant humanized IgG1 kappa isotype monoclonal antibody fragment	Recombinant antihemophilic factor
Trehalose content (mg/mL)	20[a]	60	100	10[a]
Other excipients	0.5 mg/mL L-histidine HCl, 0.32 mg/mL L-histidine, 0.09 mg/mL polysorbate 20	5.8 mg/mL sodium phosphate, monobasic 1.2 mg/mL sodium phosphate, dibasic 0.4 mg/mL polysorbate 20	10 mM histidine HCl, 0.01% polysorbate 20	108 mEq/L sodium, 38 mg/mL mannitol, 12 mM histidine, 12 mM Tris, 1.9 mM calcium, 0.15 mg/mL polysorbate 80, 0.1 mg/mL glutathionine

[a] Concentration of trehalose upon reconstitution.

choice because it can be sterilized by autoclaving without the browning associated with conventional parenteral formulations[144] and by γ-radiation.[145]

Furthermore, trehalose has been examined in great detail in research applications, including its use as a stabilizer of proteins, enzymes, and tissues, and as a therapeutic for a variety of diseases, including osteoporosis and Huntington's disease (HD) (Table 4). Each one of these will be examined in more detail below.

Research Applications

Proteins and Enzymes

With the advancement of recombinant technology, an increasing number of proteins, monoclonal antibodies, and enzymes are being synthesized each year. Most, if not all, of these macromolecules are inherently unstable and require the presence of stabilizers to provide sufficient storage stability in order to be considered a viable therapeutic product. In solution, co-solutes such as trehalose have been demonstrated to enhance the stability of a variety of macromolecules through preferential hydration.[78–80] For example, the addition of 1.5 M trehalose to the solution at pH 7 resulted in an increase in T_m of 10.9°C, 15.6°C, 14.4°C, and 8.6°C for RNase A, α-CTgen, lysozyme, and cytochrome *c*, respectively.[79]

Although the cost of production would be lower for liquid protein therapeutics, the stability of the macromolecule may be insufficient (even in the presence of stabilizers) and require preparation in the dried state, most typically achieved by freeze drying. The protein structural conformation is highly sensitive to its environment (i.e., pH, hydration level, etc.) and may denature irreversibly upon lyophilization. Furthermore, proteins can be susceptible to degradation by a variety of mechanisms (i.e., deamidation, oxidation, aggregation, etc.).[69,215–217] There are several other deleterious effects encountered during the freeze drying process, including ice formation, cold denaturation, and increased concentration, all of which must be countered with the use of appropriate stabilizer(s).[217–224] Furthermore, during storage, the suppression of various degradation processes, including deamidation, hydrolysis, and oxidation, requires the macromolecule to be restricted in mobility and limited in exposure to various media (including water and oxygen). Both can be accomplished with the use of amorphous, glass forming-excipients, such as trehalose.[25,225–228]

As discussed in the direct interaction mechanism section, trehalose has been utilized to enhance the storage stability of lysozymes and PFK.[67] Carpenter and Crowe[67] demonstrated that the addition of trehalose inhibited the shift in the frequency of amide I and II bands of lysozyme, which are observed upon desiccation in the absence of trehalose. This suggests that the dried protein in the presence of trehalose adopts the same configuration as it does in the hydrated state. The amount of trehalose addition must be optimized, as too low of a concentration results in insufficient replacement of hydrogen bonds (lost by the removal of water) and too high of a concentration can lead to trehalose crystallization, again leading to insufficient hydrogen bonding to the protein surface. Similarly, the storage stability of freeze dried HLL was improved in the presence of trehalose[69] and was attributed to its ability to maintain the native structure upon dehydration and high T_g. Furthermore, the stability of HLL, both in terms of percent monomer

Table 4. Summary of Protective Effects of Trehalose Compared with Sucrose and a List of Pharmaceutical Applications

Protective effects	Trehalose	Sucrose	References
Preservation of lyophilized protein activity [*Humicola lanuginosa* lipase (HLL)][a]	100%	<30%	69
Preservation of dried restriction enzymes at 37°C[b]	100%	0%	25,37
Liposome stabilization (inhibition of liposome fusion)[c]	~100%	80%	92
Preservation of bacteria (*E. coli*, *B. thuringiensis*)[d]	70%/57%	56%/44%	97
Suppression of polyglutamine aggregation[e]	25%	15%	146

Application	*Examples*	*References*
Proteins	β-Galactosidase, insulin, lactate dehydrogenase, phosphofructokinase, ribonuclease A, recombinant human interleukin-11, Factor VIII, human growth hormone, IgM and anti-IgE antibodies, recombinant human relaxin	62,67,72,89,147–157
Cells	Sperm cells, platelets, carrot and tobacco cells, *Lactobacillus acidophilus*, Islets of Langerhans, skin grafts, bovine embryos, canine lungs and trachea	65,158–171
Virus	Live attenuated measles virus, inactivated influenza virus, 17D yellow fever virus, Newcastle disease virus, Rinderpest and Peste des Petits ruminants viruses, respiratory syncytial virus, diphtheria/tetanus	172–178
Medical application	Osteoporosis, organ transplant, dry eye, cavity, cancer, dialysis, Huntington's disease	141,161,179–199
Pharmaceutical application	Quick-dissolve tablet, controlled release, pulmonary delivery	32,200–214

[a] Recovery of hydrolytic activity of recombinant HLL stored at 60°C for 3 months. The concentration of both trehalose and sucrose was 300 mM and that of the protein was 4 mg/mL.

[b] Restriction enzymes PstI and HindIII were dried at room temperature in the presence of trehalose or sucrose and then stored at 37°C for 35 days. Remaining activity was assessed by the ability of the enzymes to cut 1 μg of bacteriophage lambda DNA at 37°C.

[c] Unilamellar liposomes composed of palmitoyloleoylphosphatidylcholine and phosphatidylserine at 9-to-1 molar ratio was prepared. For every mole of lipid, there were 0.75 and 0.25 mol of carbohydrate and isocitrate, respectively.

[d] *Escherichia coli* (*E. coli*) DH5α and *Bacillus thuringiensis* (*B. thuringiensis*) HD-1 were stabilized by freeze drying in the presence of trehalose or sucrose (100 mM).

[e] With respect to phosphate buffered saline control on a model protein containing 35 glutamine repeats. The concentration of trehalose and sucrose was 50 μM.

content and percent activity, was reported to be superior for trehalose in comparison with sucrose, with the higher T_g of trehalose playing an important role. If trehalose alone is insufficient as a stabilizer, it is possible to utilize a combination of trehalose with a high-molecular-weight carbohydrate, such as dextran; dextran alone is also insufficient due to steric restrictions and phase separation despite its high T_g. This approach was utilized in improving the storage stability of actin co-lyophilized with 5% trehalose,[229] in which the amount of active protein following 8 weeks of storage at 60°C was improved from 50% to 74% upon the inclusion of 1% dextran. In another example, the recovered activity of lyophilized PFK and lactate dehydrogenase was significantly improved upon the addition of 1% polyethylene glycol (PEG) to trehalose;[219] at low trehalose concentrations (<100 mM), the recovered activity of both enzymes was low (<20%); however, upon the inclusion of 1% PEG, the activity was improved to at least 90% at 50 mM trehalose concentration. In another example, the addition of hydroxyethyl starch was shown to improve the stability of recombinant human interleukin-11[150] and ribonuclease A,[149] both lyophilized with trehalose. Furthermore, trehalose was shown to be less likely to phase separate from the mixture than sucrose.[149]

DNA restriction endonucleases EcoRI, Bg1II, PstI, and HindII all withstood prolonged exposure to temperature as high as 70°C, if dried in the presence of trehalose (15%), but not with other sugars.[25] Furthermore, the restriction enzymes were still able to act accurately on DNA following storage at 70°C for 35 days.[226]

For a more detailed description of protein stabilization, the reader is referred to the work of Timasheff and coworkers, Pikal and coworkers, and Carpenter and coworkers.[78,80,85,86,222,230–242]

Liposomes

Liposomes have been studied as a model cell membrane and as a vehicle for targeted drug delivery. The structural components of typical lipsomes include phospholipids, cholesterol, and proteins. As a model cell membrane, liposomes have been studied

extensively to understand the effects of desiccation on the integrity/stability of the membrane by examining the shift in phase transition temperature (T_m),[64,243,244] retention of encapsulated dyes,[93,245–247] and the degree of phase separation.[94,248] The effects of various lyoprotectants, including trehalose, on the structural integrity of liposomes have been examined. Some of the main observations include the lowering of T_m,[34,243,249] inhibition of aggregation/fusion[249,250] and inhibition of phase separation,[94,248] all of which contribute to the enhanced stability of liposomes. T_m indicates the temperature at which the membrane lipids undergo a transition from the gel phase ($T < T_m$) to the liquid crystalline phase ($T > T_m$). The desiccation of lipids increases the packing between the lipid molecules due to the removal of water, thus stabilizing the gel phase and increasing the T_m. For DPPC, the T_m increases from 42°C in the hydrated state to beyond 100°C upon desiccation.[251] Addition of trehalose prior to desiccation resulted in the lowering of the T_m to approximately 25°C (below the T_m of the hydrated state),[64,251] and this has been ascribed to the ability of trehalose to preserve the membrane structure (present in the hydrated state) during desiccation. In another example, trehalose was demonstrated to provide complete protection of freeze dried liposomes, consisting of palmitoyloleoylphosphatidylcholine (POPC) and phosphatidylserine (PS), as measured by the retention of entrapped marker, isocitrate.[92] At 1 g trehalose/g lipid, maximal stability was observed. In contrast, sucrose was only able to confer approximately 80% protection at the highest concentration examined (Table 4). The protective efficacy of trehalose, however, relies on the sugar being present on both sides of the liposomal structure. For large unilemellar vesicles composed of egg phosphatidylcholine, the amount of $^{22}Na^{+}$ entrapped within the liposomes was significantly higher (84%) for those air-dried with trehalose on both sides of the membrane than for those with trehalose present only on the outside of the membrane structure (17%).[246] If the liposome is studied as a model for the cell membrane, what this result suggests is the importance in incorporating trehalose to the intracellular compartment for effective preservation. Furthermore, the addition of trehalose inhibited the fusion of unilamellar vesicles. Addition of trehalose at a 5:1 weight ratio to lipid resulted in complete inhibition of fusion, as measured by dynamic light scattering; in the absence of the sugar, the vesicle size increased from 100 nm to more than 3000 nm.[34] The mechanisms by which trehalose stabilizes liposomes include contributions from both direct (i.e., water replacement) and indirect (i.e., vitrification) interactions. Among the oligosaccharides, trehalose has the highest ability for hydration, which suggests that trehalose may be stabilizing the bilayer structure of liposomes by ordering the water molecules around the membrane.[252] There are also numerous reports examining the effects of trehalose on the stability of liposomes in solution.[253–255] For a more comprehensive description of membrane stabilization, the reader is referred to the work of Crowe and coworkers.[9,12,21,22,249,250,256,258]

DNA

Along with lipids and proteins, the other essential biological target for stabilization is the genomic material, DNA. The effects of trehalose on the stability of DNA structure and its activity have been reported, but the effects appear to be indirect. Trehalose was observed to inhibit the strand scission of Φχ 174 DNA in the presence of $CuCl_2$ and oxidative linoleate,[258] most likely through its direct interaction with the Cu ion and the unsaturated C—C bond in linoleate, thereby removing the causative agent(s) for DNA instability. Trehalose was also shown to suppress the formation of HPO (the breakdown product of fatty acids), which has been reported to damage DNA.[259] Commercially, DNA has been stabilized by Biomatrica® (San Diego, CA), employing a technology involving air drying (SampleMatrix®).

Cells and Tissues

Various types of cells and tissues have been stabilized using trehalose. This fact may not be surprising as the structural components of cells (i.e., lipids, proteins, and DNA) have all been stabilized, as described above. For example, the viability and function of mammalian insulin-producing cells were improved during freezing and long-term storage upon trehalose incorporation.[260] The isolated islets of Langerhans, which are used in the treatment of clinical and experimental diabetes, were also reported to be successfully cryopreserved using trehalose.[158] The survival rates of skin grafts, taken from rabbit ear, were reported to be enhanced for those stored in trehalose solution, following prolonged storage at 4°C.[160] In addition, the viability of lyophilized bovine embryos was increased significantly upon trehalose addition.[159] Furthermore, canine lungs preserved in trehalose demonstrated significantly greater function and histology compared with those preserved in glucose;[161] long-term cryopreservation at −85°C in trehalose-containing solution resulted in viable tracheal graft tissue, and in reduced transplant rejection without immunosuppressive therapy.[162]

Trehalose was also shown to be effective as a cryoprotectant for carrot and tobacco cells, as long as it was applied as a pretreatment to cells.[163] This indicates that either the cells need to be adapted prior to freezing (i.e., osmoadaptation) or that trehalose must be taken up within the plasmalemma to be effective. There are also several reports demonstrating the importance of trehalose in the intracellular

compartments of cells to increase their viability to freezing and drying.[171,261] Trehalose imparts stabilizing effects indirectly as well, for example, by modifying the growth rate and morphology of ice crystals during freezing. Ice crystal growth in trehalose solution is retarded, and the fully developed ice crystals are reported to be shaped like flower petals, with rounded edges, as compared to traditional ice structures, which have jagged edges.[44] The morphology of the ice crystals may have an effect on the amount of physical damage imparted onto membranes and cells.

Viruses

The stabilization of viruses, mainly for their use as vaccines, has been examined extensively in efforts to improve their shelf life and heat stability. All vaccines, particularly of live attenuated origin, are either frozen or stored under refrigeration. Furthermore, various vaccine products are processed by lyophilization to enhance their storage stability. In a recent publication, Ohtake et al.[172] utilized a spray drying method to increase the shelf life of live attenuated measles virus to 10 weeks at 37°C in comparison with 5 weeks for Rimevax® (from package insert, GlaxoSmithKline, Middlesex, United Kingdom). The formulation contained several components, but one of the key ingredients was trehalose, which served to stabilize the virus through hydrogen bonding and vitrification. In another example, whole inactivated influenza virus was co-lyophilized with trehalose (99% weight) and delivered to rats via intranasal (IN) administration.[173] The storage stability of the lyophilized influenza virus was significantly improved in comparison with its liquid form; retention of 100% hemagglutinin (HA) titer in comparison with less than 20% following 12 weeks of storage at 25°C for lyophilized and liquid influenza virus, respectively. Furthermore, the nasal IgA titers were comparable for rats immunized IN with either the liquid or lyophilized influenza vaccine. In another application, trehalose was used to prepare a heat-stable diphtheria/tetanus vaccine adjuvanted with alum.[178] Gribbon et al. prepared the stabilized vaccine by ambient drying (Q-T4 technology), which circumvented the problems associated with freezing aluminum hydroxide (i.e., aggregation and precipitation). The activity of the trehalose-stabilized vaccine was maintained above 90% following 35 weeks storage at 45°C, whereas the control vaccine (without trehalose) demonstrated less than 5% activity following 2.5 weeks of storage at the same temperature. Furthermore, analysis of aluminum hydroxide sedimentation revealed that trehalose-stabilized vaccine maintained 90% of the values of the fresh suspension control, whereas those stabilized in sucrose demonstrated only 50%, following 4 weeks of storage at 45°C. Trehalose was also utilized to stabilize 17D Yellow Fever virus,[174] Newcastle disease virus,[175] and Rinderpest and Peste des Petits ruminants viruses.[176] All of the viruses were dried by different techniques, including freeze drying, spray drying, and foam drying; however, they all demonstrated the efficacy of trehalose in conferring stability to a wide variety of stresses. It should be noted that trehalose was successful in providing protection on significantly different time scales (i.e., dehydration occurring in days and microseconds for freeze drying and spray drying, respectively). Trehalose was also examined as a stabilizer in solution for respiratory syncytial virus.[177]

Microorganisms (Bacteria, Fungi)

Trehalose has been found to be synthesized in several bacteria in response to osmotic stress,[262–265] thus it is a natural choice to be examined as a stabilizer. For example, *Pseudomonas fluorescens* EPS62e that contained higher levels of intracellular trehalose, as a response to salt stress, demonstrated higher desiccation tolerance.[266] Leslie et al.[97] utilized trehalose (100 mM) to stabilize both *Escherichia coli* (*E. coli*) DH5α and *Bacillus thuringiensis* (*B. thuringiensis*) HD-1 by freeze drying. In the absence of any protectants, only 8% of *E. coli* and 14% of *B. thuringiensis* survived, whereas in the presence of 100 mM trehalose, the survival rate increased to 70% and 57%, respectively. Sucrose was also effective in increasing the stability, but less so than trehalose. The decrease in viability can be attributed to damages to the membrane integrity and protein structure during dehydration, and subsequent rehydration. By examining the effects of dehydration on the membrane phase transition temperature (T_m), the authors noted that the T_m increased from 10°C to 50°C for *E. coli*. In the presence of trehalose, however, the T_m of the lyophilized *E. coli* was maintained close to the value of the hydrated bacteria (10°C), thus maintaining the fluidity of the membrane similar to that present in the hydrated state. The effects of drying on membrane proteins were determined by observing the changes in the frequency of amide I and II bands, which indicate alterations in the conformation/structure of proteins. In both *E. coli* and *B. thuringiensis*, freeze drying caused the amide II band to shift in frequency compared to that reported in the hydrated state. More specifically, the amide II band shifted from 1543 to 1533 cm^{-1} in *E. coli*, whereas for *B. thuringiensis*, the wave number shifted from 1546 to 1533 cm^{-1}. In the presence of trehalose, no changes in amide II band were observed, suggesting that the protein conformation was maintained during desiccation. Leslie et al.[97] also noted that sufficient amount of sugars are necessary within bacteria to confer stability, and uptake was made possible by lowering the temperature of the bacterial suspension through the T_m of the bacterial membrane to allow for passive transport of trehalose

(i.e., at T_m, membrane integrity is at its lowest point, facilitating transport through passive diffusion). Finally, the storage stability of freeze dried *E. coli* and *B. thuringiensis* was examined by exposing the samples to light, air, and humidity. No decrease was observed in either bacterium co-lyophilized with trehalose, whereas sucrose was deemed to be ineffective. In another application, foam drying of *Salmonella typhi* bacterial Ty21a vaccine with trehalose resulted in significant stabilization of the vaccine;[267] addition of trehalose resulted in reduction of process-associated loss from 2.2 to 0.4 $\log_{10}$ CFU/mL and storage loss was minimized from 7.4 to 2.3 $\log_{10}$ CFU/mL, following 4 weeks of incubation at 37°C. Trehalose was also reported to act as a free radical scavenger for *Saccharomyces cerevesiae*.[5]

Medical and Pharmaceutical Applications

Organ Transplant

The combination of donor shortage and the long-distance transportation often required for available tissues and organs highlight the importance of an effective preservation methodology. Similarly, the preparation of graft that is both fresh and appropriate, especially on short notice, is very difficult, if not impractical.[162] Even a minute improvement in the storage stability of these labile biologicals would be of paramount importance and value.

New organ preservation solution was developed at Kyoto University,[162] named extracellular-type trehalose-containing Kyoto (ET-Kyoto) solution, and was successfully used in clinical lung transplantation; the patient underwent bilateral lobar lung transplantation on cardiopulmonary bypass from living donors, and each lower lobe was flushed using 2 L of ET-Kyoto solution. In comparison with Euro-Collins and University of Wisconsin solutions, which are the primary solutions used for clinical lung transplantation, ET-Kyoto was demonstrated to be equal or better in its preservative effect. Trehalose is present at 41 mg/mL and is one of the major constituents (>50 wt %) in the ET-Kyoto solution.

The common difficulty encountered during lung transplantation is the formation of edema of the endothelium within the pulmonary vessels caused by ischemic injuries. An effective preservation media would be able to stabilize the cell membranes, resulting in the suppression of cellular edema occurrence, potentially leading to prolonged storage.[161] In one study, all transplanted lungs preserved in trehalose-containing Euro-Collins solution (35 mg/mL) demonstrated normal histology, whereas those preserved in Euro-Collins solution (with glucose in place of trehalose) developed severe pulmonary edema.[161] The value of partial pressure of oxygen in arterial blood (PaO_2) in the transplanted lungs was monitored at several time points following reperfusion. The lungs that were preserved in the trehalose-containing solution demonstrated PaO_2 values (130 min following reperfusion) similar to that observed in the preoperative group (264.9 ± 26.2 and 281.8 ± 10.6 mmHg, respectively), whereas those stored in glucose solution demonstrated a significantly lower PaO_2 value (134.7 ± 49.4 mmHg).[161] The effectiveness of trehalose in improving the stability of lung tissues is thought to derive from its ability to stabilize the cell membrane, thereby suppressing damage from pulmonary ischemia and low temperature, both of which are essential for long-term preservation.

Trehalose-containing solution was utilized in another application to improve the storage stability of trachea and its function upon transplantation in dogs.[197] Two of the major problems encountered during tracheal transplantation are rejection and preservation. Trachea were removed from donor dogs, and then immersed in various preservative media. The trachea were cryopreserved at −85°C for 285 ± 28 days.[197] All animals survived more than 2 months, following transplantation, and none of the grafts demonstrated stenosis or tracheomalacia. On the contrary, in the control group (stored in Euro-Collins solution at 10°C for 16–17 h), most of the animals died within 1 month due to tracheal stenosis caused by rejection.[197] Most candidates for tracheal transplantation possess malignant diseases, and immunosuppressants cannot be given readily after operation. Thus, the ability of trehalose to increase the storage stability and to suppress rejection is an attractive proposition.

The solution has also been successfully used with lung transplantation in other animal models.[191–195,198,199] Trehalose has also been successfully used to cryopreserve human pancreatic endocrine tissue[260] and skin free-flaps of rabbit ears.[160]

Osteoporosis

Osteoporosis is fast becoming a major problem, particularly in postmenopausal women, causing considerable pain and immobility due to bone fractures. Osteoporosis results from an imbalance in bone formation and resorption (i.e., destruction). Although there are several causes for osteoporosis, the lack of estrogen production after menopause is considered to be the major contributor.[188] Estrogen replacement therapy has been demonstrated to be effective in preventing bone loss in animals and humans;[268–269] however, the continuous administration of estrogen is accompanied, at times, with severe adverse side effects.[270]

The effects of trehalose on bone resorption were studied using ovariectomized (OVX) ddY mice as a model of osteoporosis caused by estrogen deficiency.[188]

In these mice, the bone weight, calcium and phosphorous contents of the femur, and the trabeculae of the tibias were all noted to decrease 4 weeks after surgery. All of these phenomena are thought to be caused by an increase in the rate of bone resorption, with respect to bone formation. Trehalose was orally administered to OVX mice five times a week for 4 weeks, and the changes in bone weight and calcium/phosphorous contents were analyzed. Bone weight loss was prevented in a dose-dependent manner. A significant inhibition of bone weight loss was observed in the group treated with 100 mg/kg trehalose compared with the OVX non-treatment group; −0.20 compared with −0.34 mg/g body weight, respectively.[188] In addition, the trabeculae of the OVX mouse tibias in the trehalose treatment group were only marginally decreased when compared with those of the control mice. Furthermore, the increase in osteoclast (OC) formation was significantly inhibited by the *in vivo* administration of trehalose; 5.3×10^3 OCs/well were observed following 100 mg/kg administration of trehalose, whereas 7.6×10^3 OCs/well were observed in the absence of trehalose.[188] Trehalose is unlikely to have an estrogen-like function, as its administration had no effect on uterine weight. Trehalose was also shown to inhibit the secretion of interleukin (IL)-6,[189] one of bone resorption-associated cytokines, and suppress the formation of tumor necrosis factor (TNF)-α, following lipopolysaccharide (LPS) injection.[271] The exact mechanism of the suppressive effects of trehalose is unknown; however, trehalose is postulated to suppress OC differentiation, which is induced by IL-6 secretion,[189] and interfere with the binding of LPS to TNF-α-producing cells.[271]

These preliminary animal studies demonstrate the potential of trehalose therapy in improving bone metabolism and preventing osteoporosis, although it is unclear how oral ingestion of trehalose can be effective, as it is readily metabolized into two glucose molecules in the intestinal tract, thus reducing the amount of trehalose in circulation, if any remains at all. As no further detail was given in the studies, it may be helpful to compare the limit of trehalose digestion in mice and the dose of trehalose administered, as well as providing comparable glucose solutions (i.e., 2× the trehalose concentration) as negative control. How this relationship translates to the doses required for human patients would also provide valuable information.

Huntington's Disease

Huntington's disease (HD) is a progressive neurodegenerative disorder. The cause of the disease is thought to be the aggregation of mutant Huntingtin protein, characterized by its abnormally long glutamine repeats. Several small-molecule compounds, including trehalose and sucrose, were screened to determine their efficacy in reducing the aggregation of the model protein, bearing an expanded polyglutamine chain.[146,272] Aggregation, as monitored by the turbidity of the protein solution, was decreased upon the inclusion of trehalose in a dose-dependent manner, with the relative absorbance decreasing to approximately 50% of the phosphate buffered saline control upon the addition of 1 mM trehalose. Furthermore, trehalose was shown to suppress the formation of aggregates in mammalian cells (mouse neuroblastoma Neuro2a cells) and to improve their viability. In all of the studies, trehalose was demonstrated to be superior to sucrose. Furthermore, oral administration of 2% trehalose solution to R6/2 transgenic mice resulted in the reduction of aggregate formation in the motor cortex, striatum, and liver (∼40–50% of the control) and in improved motor function as well as their survival. The efficacy in inhibiting polyglutamine aggregation, coupled to its safety, may place trehalose as one of the leading candidate as a therapeutic compound for the treatment of HD and other polyglutamine diseases.

In other related applications, oral administration of trehalose was shown to suppress the aggregation of polyalanine-rich proteins, as a model for oculopharyngeal muscular dystrophy (OPMD), and to alleviate the symptoms of OPMD in transgenic mice.[273] In addition, trehalose was reported to be effective in reducing the aggregation of β-amyloid peptides,[274] which is a key step in the pathogenesis for Alzheimer's disease.

Although *in vitro* studies corroborate the effect of trehalose in suppressing peptide aggregation, their effects *in vivo* are more difficult to interpret, as trehalose administered orally should have been degraded into two glucose molecules for absorption in the small intestine. For most of the studies, glucose was examined as a control and demonstrated significantly less effect in comparison with trehalose. Thus, how trehalose administration resulted in the reduction of aggregate formation in the brain of rats[146] is still a mystery.

Other Medical Applications

Trehalose, or one of its derivatives, has also been examined and used in several other medical applications including peritoneal dialysis system,[187] therapeutic for cancer,[182–186] preservation of sperm cells,[164–166] platelets[65,167–170] and other mammalian cells,[171] treatment for dry eye syndrome,[179] and as a prophylactic for cavity formation.[181] The results from these studies, although mostly in animals or *in vitro*, appear promising and may warrant further examination by companies, particularly if the results from clinical trials are positive, as is the case with dry eye syndrome.[179,275,276] Cellphire (Rockville, MD) is currently developing lyophilized platelets for hemostasis and wound-healing therapies.

Use in Solid Dosage Form

The use of trehalose in therapeutic products, thus far, has been limited to its use as a stabilizer. The sugar does, in fact, possess several unique physical and chemical properties that may enhance its use as an ingredient in solid dosage forms. For information on crystalline or polymorphic properties of trehalose, the reader is referred to the section entitled *Unique Properties of Trehalose*. In the United States, there are currently no trehalose-containing pharmaceuticals in any tablet products; however, in Japan, trehalose is included in Sawachion (Sawai Pharmaceuticals), Imidapril (Ohara Pharmaceutical Co., Ltd.), and Bio-Three HI (Towa Pharmaceutical Co., Ltd.) as a bulking agent and, in some cases, as a binder. In addition, the number of publications examining the use of trehalose in solid dosage form is quite limited[277,278] and pipeline products containing trehalose in solid dosage form are undisclosed. There are, however, a number of patents claiming the use of trehalose in solid dosage applications, in particular as a diluent or a modifier of the physical properties of quick-dissolving tablets for administration to the buccal cavity (Table 2).

Trehalose has been included as a bulking agent and/or a binder in a number of tablet applications.[32,201,202,204,205] This is mainly due to its stability toward high temperature and hydrolysis (i.e., Maillard reaction) and its high moldability. A number of bulking agents, including lactose, mannitol, and calcium phosphate, are used to increase the bulk of the tablet for effective compression. Lactose has been used frequently as a tableting excipient. However, as it can be reduced readily, its use in the presence of amine bases or salts is not recommended. Trehalose, on the contrary, does not react with amino groups, and is highly resistant to hydrolysis. In addition, the substitution of lactose with trehalose can alleviate the fears of patients suffering from lactose intolerance; even though the amount of lactose used in tablets is comparatively low, the patients may find psychological relief from its complete removal. Furthermore, tablets produced with anhydrous trehalose do not suffer from excess hardness and readily dissolve under appropriate conditions, unlike those prepared with amorphous lactose. The aforementioned properties of trehalose should also be applicable and extended to pharmaceuticals prepared in capsules. In another application, trehalose was used to spray coat a tablet[206] or bitter-tasting active agent.[207] Furthermore, trehalose may offer structural stability as a bulking agent (as in lyophilized formulations), particularly for applications involving rapidly disintegrating tablets. These tablets are typically formed from blends containing volatile salts such as ammonium bicarbonate. The salts are removed under vacuum at elevated temperatures for an extended period of time, resulting in a highly porous structure that rapidly dissolves due to its high surface area.[32] The conditions required for tablet preparation may preclude the use of certain excipients such as sucrose, which have low T_g and propensity for hydrolysis. In other tablet processing methods, the hardness of the tablets is increased by their treatment with humidification and drying; humidification is typically conducted between 30 and 100% relative humidity (RH) at 20–30°C in a thermostatic chamber followed by drying at 20–60°C.[200–203] The purpose of these treatments is to convert the sugar from the amorphous state to the crystalline state, thus increasing the strength and stability of the tablet. The physical and chemical stability of excipients, particularly sugars that are reducing or prone to hydrolysis, may not be amenable to such treatments, particularly in the presence of amine-containing biotherapeutics.

Trehalose has also been used to modify the hardness and friability of tablets as well as their porosity and dissolution time.[203,208,209] Furthermore, in tablet processing applications, a binder is necessary to hold the components together to give strength to the final dosage form. Compared with the binders that have been typically used, trehalose has the advantage due to its high moldability and enhanced physical/chemical stability, as explained above (high moldability refers to an excipient that results in tablet hardness of $\geq$2 kp upon tableting 150 mg of the material under a pressure of 10–50 kg/cm^2).[200,210] In one example, trehalose has been used as a binder in a quick-disintegrating tablet that contains sustained-release fine particles.[202] In another example, trehalose was used as the main ingredient and stabilizer in tablets produced by S-1 spray freezing process.[211] Trehalose was reported to be a superior choice to mannitol, particularly due to its high solubility at room temperature, ability to be lyophilized without glass collapse, and effectiveness as a stabilizer in the dried state. In addition, trehalose-containing tablets dissolved rapidly in aqueous solutions and yielded high tablet-to-tablet reproducibility.

Trehalose has also been described in several other patent applications for its use as a drug delivery vehicle. In one application, trehalose was used in conjunction with a biodegradable polymer (i.e., polycaprolactone) for controlled-release delivery of a peptide or protein;[212] trehalose was chosen due to its ability to form a glassy matrix and because its T_g is higher than the melting point of the biodegradable polymer, wherein mixing the two components, the glassy matrix is made feasible. In other applications, trehalose was used as a dissolvable vehicle for controlled delivery of particles[213] and also as a bulking agent for dry powder containing N-terminal fragment of parathyroid hormone intended for pulmonary delivery.[214] The number of scientific publications describing the use of

trehalose in the solid dosage format may not be abundant, yet the number of patent applications appears to be increasing steadily. With all the advantages that trehalose possess over the traditional excipients (i.e., lactose, sucrose, and mannitol), it may just be a matter of time before trehalose-containing tablets appear on the counter at a near-by pharmacy.

USE IN THE FOOD INDUSTRY

In Japan, trehalose is consumed in large quantities and is present in various food products. Tens of millions of pounds of trehalose have been used in the food industry since 1995, and currently, the production estimate is 25,000–30,000 tons/year.[7,27,49] Trehalose is present in over 8,000 products with confectionary products consuming a large share of its total use. The popularity of trehalose may be due to its lower sweetness and longer persistence (in sweetness) in comparison with sucrose.[279] No definitive explanation for the difference in perceived sweetness is given; however, the difference in the binding capability of the sweet taste receptor for the two sugars has been put forth as a possible explanation (due to the differences in size of the sugar-associated water cluster and structural configuration).[279–281] Furthermore, in a panel study, the perceived sweetness of trehalose was determined to increase faster relative to sucrose, and 85% of the tested subjects preferred the taste of trehalose compared with sucrose.[7] Besides confectionary products, trehalose is present in beverages, processed vegetables and fruits, baked goods, processed seafood, frozen food products, and refrigerated items.[7] In the United States, trehalose can be found in frozen shrimp products (Grilled Shrimp Classic and Grilled Shrimp Scampi) from Gortons, Inc. (Gloucester, MA) and in a number of nutritional/energy-boost products including Accelerade® and Accel Gel® (Mott's, Rye Brook, NY), CARB Max and GAIN Max (Sport Pharma, Concord, CA), Glycomaize (Optimum Nutrition, Aurora, IL), and Endurathon and Myoplex Deluxe (EAS, Abbott Laboratories, Abbott Park, IL). New products are also in development and include vegetable/fruit chips, bread made from rice powder, and meringue, all benefiting from, and made feasible by, the unique properties of trehalose.[49] As described previously, trehalose occurs naturally in many of the widely consumed food products including mushrooms,[282] bean products,[283] honey,[26]invertebrates such as lobster, crab, and shrimp,[26,283] yeast products such as bread,[284] beer,[285] wine,[286] vinegar,[287] and honey,[288] and certain seeds.[289] Trehalose content of dry solid material can range from 10 to 23% for mushrooms and from 7 to 11% for Baker's yeast.[283] Thus, trehalose is present in a wide variety of both processed and natural food products.

Trehalose has gained regulatory approval in many countries, starting with the UK as a cryoprotectant for freeze dried food product at concentrations of up to 5%.[7] It was then approved as a food ingredient in Korea and Taiwan in 1998, with no use limits, and soon after in 2000, trehalose was deemed safe (generally regarded as safe or GRAS) by the US Food and Drug Administration (FDA). Regulatory approval as a novel food ingredient was granted in Europe in 2001.

As can be expected from the prevalence of trehalose in nature, humans have evolved specific intestinal enzymes to digest trehalose, similarly to other disaccharides. Upon ingestion, trehalose is enzymatically hydrolyzed in the small intestine by a trehalose-specific disaccharidase (trehalase) into two D-glucose molecules, which are subsequently absorbed and metabolized.[290] Although there are no gender- or age-associated differences in trehalase activity,[291] there are reports of ethnic differences in the ability to ingest trehalose; slightly lower capacity to tolerate trehalose was noted in Asian populations.[292–295] Despite these differences, safe human consumption of trehalose in doses up to 50 g has been demonstrated;[292] thus, there appears to be no barrier for the inclusion of trehalose in future food products.

Food Preservation

The drying of foodstuffs is an attractive approach not only for ensuring stability but also for reducing the weight and volume of the product (due to the removal of water), which may lessen transport and storage requirements. As for biologicals, as described above, there are a number of issues associated with drying foodstuffs, including reduction of visual appeal, smell (upon reconstitution), and quality. Upon the addition of trehalose prior to air drying, a wide variety of food products were protected from denaturation, and the sugar prevented the loss of aromatic volatiles, which confer fresh food products their characteristic aroma and flavor.[25] Blended fresh eggs dried with trehalose (at 40–50°C) resulted in an odorless yellow-orange powder that can be stored at room temperature and rehydrated easily, resulting in a product virtually indistinguishable from fresh eggs.[110] Superoxide dismutase (SOD)-like activity of vegetables was also maintained upon drying fresh vegetables with trehalose. Six hundred grams of minced carrot and 66 g of trehalose were mixed and dried *in vacuo* at 40°C for 40 h. The dried carrot–trehalose mixture was powdered and then stored at 40°C for 7 days. The remaining SOD-like activity was higher than that of the carrot powder alone (Table 5), and the degree of stabilization was reported to be highly dependent on the ratio of trehalose-to-vegetable used.[296] A similar protective effect of trehalose in maintaining the SOD-like activity was observed for several other vegetables, including cucumber, spinach, and onion.[296]

Table 5. Summary of Protective Effects of Trehalose Compared with Sucrose in Food Products

Properties	Trehalose	Sucrose	References
Activity Preservation / Stabilization			
Preservation of SOD-like activity of carrot powder[a]	65%	28%	48,259,296
Protection of vitamin E to heating[b]	~75%	<5%	27
Suppression of AAPH-induced radical oxidation of linoleic and α-linolenic acid to generate HPO[c]	55%, 50%	−3%, −3%	74
Suppression of Foul Odor			
Suppression of propanal formation from α-linolenic acid[d]	87%	5%	24,27,48,76,259,298
Suppression of 2,4-decadienal formation from linoleic acid[e]	88%	4%	24,27,76
Suppression of DMS and DMDS formation upon heating of milk[f]	66%, 100%	−11%, −95%	27,44
Suppression of TMAO degradation[g]	40%	–	44,75,76
Suppression of diacetyl formation[h]	99.8%	95.5%	299
Food Quality and Safety			
Suppression of Mg ion leakage from spinach and pork meat upon heating[i]	53%, 59%	0%, 0%	27,49
Suppression of starch aging[j]	88%	47%	44
Acrylamide formation[k]	0 mg/mol Asn	98 mg/mol Asn	27,259
Prevention of Maillard reaction (Browning Index)[l]	0	>6	297
Preservation of aroma[m]	effective	–	25,110

[a] 10 wt % of each sugar was mixed with minced carrot and then dried at 40°C for 64 h. The dried powder was stored at 40°C for 7 days. Superoxide dismutase (SOD)-like activity remaining upon storage without sugar addition was 11%.

[b] Amount of vitamin E (α-tocopherol) remaining following heating for 1 h at 100°C; solution contained 100 mg vitamin E, 100 mg saccharide, and 500 mg cellulose. In the absence of sugars, the amount of α-tocopherol remaining was approximately 20%.

[c] The level of 2,2′-azobis 2-amidinopropane dihydrochloride (AAPH)-induced radical oxidation of linoleic and α-linolenic acid was determined 24 h after heating the solution at 40°C in the presence of 29.2 mM saccharide. The values shown in the table are relative to the amount of HPO produced in the absence of sugars. Negative values indicate enhanced production with respect to control experiment.

[d] Suppression of α-linolenic acid decomposition (i.e., formation of volatile aldehydes such as propanal) upon the addition of saccharides to a boiling solution compared with control (no sugar). Butanal and hexanal formation was also suppressed upon trehalose addition.

[e] Suppression of 2,4-decadienal formation from linoleic acid upon boiling in the presence of trehalose and sucrose, in comparison with control (no sugar).

[f] Milk was heated for 1 min at 130°C and the amount of dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) was quantified and compared with that of control, which contained no sugar. Negative values for sucrose indicate enhanced formation of DMS and DMDS.

[g] 5% trehalose solution was added to 10 g of mackerel meat and boiled for 15 min. Degradation products of trimethylamine-*N*-oxide (TMAO), trimethylamine (TMA), and dimethylamine (DMA) were analyzed and quantified by GC.

[h] 10 wt % sucrose or trehalose was added to cacao and heated at 100°C for 5 min. The composition of the headspace gas (HSG) was analyzed by GC. Cacao heated without any sugars released 8.9 μg/mL of diacetyl.

[i] Spinach (20 g) or pork meat (30 g) was boiled for 5 min in each 10% saccharide solution. The amount of eluted Mg was quantified by absorption spectrophotometry and compared with the amount of Mg eluted out in the absence of saccharides.

[j] 2% starch solution was mixed with 12% trehalose solution and stored at 4°C for 12 h. The amount of aging was determined by the increase in solution opacity. The amount of suppression is with respect to the opacity of the starch solution in the absence of trehalose.

[k] Amout of acrylamide formation in a mixture containing asparagine and saccharide, both at 0.1 mmol concentration, was measured by GC. The solution was heated at 150°C for 20 min.

[l] Browning index is defined as the absorbance at 445 nm × dilution factor.

[m] Retention of aroma was quantified by analyzing the headspace content of volatiles by GC, upon reconstituting vacuum-dried fruit (banana and mango) in the presence of 10 wt % trehalose. Chromatogram was compared with that of fresh fruit and that dried in the absence of trehalose.

Sucrose, on the contrary, was less effective in preserving the SOD-like activity upon drying, followed by storage at elevated temperatures; 28% of pre-dried activity was preserved with sucrose compared with 65% for trehalose (Table 5). Trehalose-dried food products were also found to contain less toxic by-products and higher nutritional content than conventionally processed foods.[25] For example, fresh banana, strawberry, mango, avocado, apple, and raspberry, which were all pureed in the presence of trehalose and dried at 25–50°C, demonstrated no detectable changes in color or other properties during prolonged storage. In the dried state, the powders did not have any aroma; however, upon reconstitution, the color, viscosity, and texture of the fresh purees were restored.[25,110] In fact, a few minutes after rehydration, the purees were reported to emit the unique aroma of the fresh fruit. The preservation of volatile aromatics can be understood by the difference in hydrophilicity between trehalose and the aromatics; trehalose is hydrophilic and its glass is not miscible with or permeable to hydrophobic volatile aromatics such as esters. Because of this incompatibility, the aromatics are trapped within the glass and are only released upon reconstitution. Contrary to the stable, nonpermeable glass produced by trehalose, the glasses of other sugars that crystallize become porous and permeable to volatile aromatics, and thus, lose the aroma of fresh products over time.[110] The dried products can also undergo degradation during storage. In one example, fresh mango was pureed and then dried with and without trehalose. The volatile aromatics released upon reconstitution were collected and analyzed by gas chromatography (GC). The mango dried with trehalose demonstrated a higher number and intensity of native volatile aromatics as well as lower amounts of degradation compounds, furfural and α-humulene.[25] These degradation compounds are characteristic products formed from the Maillard reaction between the naturally occurring sugars and proteins. Thus, trehalose not

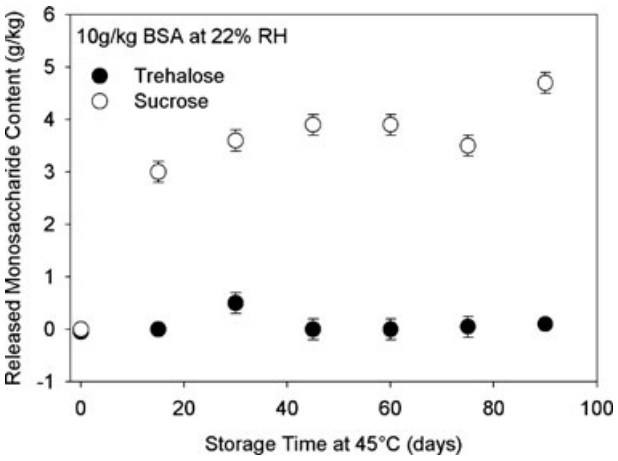

Figure 6. Progression of Maillard reaction as monitored by the increase in monosaccharide content in a lyophilized mixture of disaccharide, trehalose or sucrose (25 mg/mL), and bovine serum albumin (BSA) present at 1 wt %. The samples were stored at 45°C and 22% relative humidity (RH) for the indicated amount of time and the glucose content was determined using an enzymatic method. Reproduced from Schebor et al.[297] with permission from Academic Press.

only preserved the volatile aromatics but also suppressed degradation. In comparison, foodstuffs dried in sucrose may not enjoy similar stability, as the progression of the Maillard reaction typically results in lowered pH and sucrose is susceptible to acid hydrolysis. In one study, the progression of Maillard reaction of bovine serum albumin (BSA) co-lyophilized with either sucrose or trehalose was monitored by examining the release of monosaccharides (due to hydrolysis) during storage at 45°C and 22% RH (Fig. 6). Although trehalose-preserved BSA demonstrated stability for up to 90 days, BSA lyophilized with sucrose exhibited hydrolysis within 3 weeks of storage. The desired poor reactivity of trehalose with amino compounds is due to its high thermostability, wide range of pH stability, and lack of reducing power, as mentioned previously. As will be described next, trehalose also masks unpleasant taste and odor in food, thus it is a superb additive for the maintenance of food quality.

Effects as an Additive

Just as sugars and salts are added during food preparation to enhance the flavor (i.e., sweetness or saltiness) and/or alter the properties of cooking media (i.e., boiling point elevation), trehalose can be included as an additive as well. As a dietary sugar, trehalose is completely nontoxic and has been reported to moderately modify the flavor of foods (i.e., suppression of bitterness, enhancement of sourness, etc.).[76,280,300] For mochi-based products (mochi is a Japanese rice cake), trehalose has been incorporated to confer protection against low-temperature and freezing stresses.[299] In addition, trehalose has been included to maintain the "moistness" of mochi-based products as well as rice. The inclusion of approximately 3% trehalose has been shown to inhibit the dehydration of rice without enhancing its sweetness.[44,299] This may allow for the preparation of rice ahead of time, without compromising its freshness. In another application, the addition of 12% trehalose resulted in the suppression of starch aging (Table 5), whereas sucrose addition resulted in only 47% protection. Application of cut vegetables in 1–3% trehalose solution for 15–30 min suppressed dehydration and discoloration (caused by oxidation). For fruit, higher trehalose concentration (10–15%) is required to observe similar efficacy.[299] Trehalose addition has also been reported to prevent freezer burn, and thus improve the quality of frozen products, by modifying ice crystal formation. Furthermore, as will be discussed in more detail in the next section, food products such as sponge cake, doughnuts, and mayonnaise can all benefit from incorporating trehalose, through its ability to suppress fatty acid degradation.[44]

Administration of trehalose has resulted in lower and slower rise in blood sugar level in comparison with glucose.[259,301,302] In addition, 90 min after oral administration, the blood sugar level decreased gradually for trehalose, compared with glucose, and the insulin level was found to be lower than that released due to glucose ingestion.[301] These observations suggest that trehalose may be used as a substitute in many sugar-containing food products, particularly for those suffering from diabetes. Furthermore, trehalose may be a viable candidate for long-lasting energy source. Besides adequate level of hydration, rigorous sports activities require the replenishment of carbohydrates, allowing for the maintenance of blood sugar at an acceptable level. Owing to the lower sugar spike (lower C_{max}) and slower decrease in blood sugar level (as well as its reduced sweetness in comparison with sucrose), trehalose has been slowly gaining interest in the nutraceutical field and finds itself in a number of products; for example, trehalose is now among one of the ingredients that can be chosen to create a personalized supplement from True Protein's webpage (www.Trueprotein.com). However, the differences among the individuals' trehalase activity must be kept in consideration, as the amount and rate of trehalose degradation can vary and result in off-target blood sugar levels.

Effects During Heating/Cooking

The application of heat to fresh products is inevitable whether making confectionary products, baked goods, or during cooking. Food products containing fat generally produce unpleasant odor upon heating and processing. Poultry, fish, white rice, and mayonnaise all produce odor (aldehydes) upon storage, but upon

the addition of trehalose, odor was reduced.[27] Lipid oxidation by heating and exposure to air is the cause of peculiar odor known as degradation odor, and it is one of the main reasons for lowering the quality of food products. Furthermore, the oxidation products of lipids are known to negatively influence human health and aging. Oxidation of unsaturated fatty acids occurs readily upon heating their dispersion in water; three types of unsaturated fatty acids, including oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3) are readily degraded upon heating. In the presence of trehalose, however, their degradation was inhibited. As described previously, trehalose is postulated to interact directly with unsaturated fatty acids (through hydrogen bonding),[259] thus providing stability and inhibiting HPO production.[27] The formation of HPO, which is the initial reaction product of linoleic acid oxidation, was suppressed by trehalose through direct interaction, as evidenced by NMR.[24] Trehalose was also found to suppress the formation of 2,4-decadienal from linoleic acid during boiling (Table 5). Trehalose can suppress fatty acid degradation not only from heat but also from free radical oxidation. The formation of HPO from both linoleic acid and α-linolenic acid, due to 2,2′-azobis 2-amidinopropane dihydrochloride (AAPH)-induced radical oxidation, was remarkably inhibited by trehalose (Table 5);[74]the inclusion of 29.2 mM trehalose in the solution containing linoleic acid resulted in the reduction of HPO formation by 55%, with respect to the control experiment conducted in the absence of sugars. Sucrose, on the contrary, was ineffective in protecting the fatty acid from AAPH-induced oxidation. In another example, 100 mg of unsaturated fatty acid was mixed with 1 mL of 5% disaccharide solution and then heated to a boil for 1 h. The volatile aldehydes in the headspace gas of the vial were measured using GC (Table 5). The generation of almost all volatile aldehydes, propanal, butanal, and hexanal, was remarkably suppressed to 10–30% of the control by the addition of trehalose[24] (Table 5). Similarly, trehalose was effective in suppressing the formation of unpleasant odor associated with cooking fish, that is, inhibition of breakdown of animal fats and oils that produce aldehydes and 2,4-decadienal.[75] In both of these cases, sucrose was not as effective as trehalose in suppressing the formation of volatile aldehydes (Table 5). The formation of TMA from TMAO in mackerel meat during boiling was also suppressed by approximately 40% in the presence of trehalose.[76] The stabilization was demonstrated to occur through the direct interaction of trehalose with TMAO via hydrogen bonding.[75] In another example, trehalose was shown to suppress the formation of sulfur-associated odor during heating of milk; DMS and DMDS are produced from the breakdown of cystine and methionine upon heating milk (Table 5). This is thought to occur through the interaction of trehalose with the amino acid groups, thereby suppressing their degradation into the sulfur compounds.[27] Although the mechanism is unclear, decreased amount of diacetyl formation resulting from heating of cacao was reported upon the addition of trehalose,[299] 0.02 μg/mL compared with 8.9 μg/mL in the absence of trehalose. As high temperature is typically used for baking cacao-based products as well as chocolate, the suppression of unwanted, buttery aroma can be avoided by the addition of trehalose (10 wt %). Although not as effective as trehalose, sucrose was also shown to reduce diacetyl formation (Table 5).

As described previously, trehalose was also reported to interact with metal ions; release of Mg from chlorophyll in spinach caused by heating was prohibited in the presence of trehalose (Table 5) and the green color of the vegetable was maintained.[27] Higher amounts of other salts, including Ca, Na, and K ions, were also retained in the trehalose-containing solution. The suppression of Mg release from pork meat was also reported when boiled in the presence of trehalose (Table 5),[27] and is thought to result from the interaction of trehalose with Mg ions, which are either bound to a protein or other compounds.[27] In both of these cases, the substitution of trehalose with sucrose resulted in the elution of Mg comparable to the amount observed in the absence of sugars. The ability of trehalose to interact with metal ions has also been reported to have a profound effect in stabilizing vitamin C, which is known to degrade readily in the presence of metal ions, such as iron and copper[48]; the addition of 100 mM trehalose was shown to suppress (∼50% or more) the degradation of vitamin C (14.8 mM) in the presence of 10 mM metal ions (Fe and Cu) upon heating at 50°C. Vitamin E (α-tocopherol) was also reported to be stabilized by trehalose upon heating for 1 h at 100°C;[27]in comparison, sucrose was ineffective in stabilizing vitamin E (Table 5). In another example, 1–2% trehalose addition was shown to inhibit dehydration and stiffening of meat during heating, without enhancing its sweetness.[299] Trehalose was also reported to inhibit the formation of acrylamide during heating and processing of food products such as potato chips and cereal. Typically, acrylamides are produced through the interaction of Asn in food products with reducing sugars. Acrylamides are known to be toxic, causing damage to the central nervous system, and to cause cancer upon long-term dosage. Upon the addition of trehalose, acrylamide production was suppressed through its interaction with glucose (and thus suppressed the glucose-Asn reaction).[27] In the case of sucrose, acrylamide was produced upon heating its mixture with Asn (1:1 mole ratio), most likely through its hydrolysis at high temperature (150°C) producing glucose and fructose.

Besides the foul odor, discoloration is another factor that negatively impacts the quality of food resulting from heating and/or processing. As described previously, trehalose can stabilize protein-based food products against prolonged heat treatment due to its resistance to Maillard reaction, thus preventing discoloration. β-carotene, contained in carrots, is known to degrade upon exposure to light or oxygen and results in loss of color of the vegetable. However, carrots dried in the presence of trehalose, followed by 1 year of storage at room temperature, have been shown to maintain their color. In its absence, the carrots lost color and degraded.[44] Thus, the addition of trehalose to food products can not only improve their shelf life but also prevent the deterioration in food quality associated with discoloration and foul odor. As the industry becomes more knowledgeable about trehalose and its use, coupled to the reduction in cost, the number of trehalose-containing products may show corresponding increase. Furthermore, it is the hope of the authors that these less known interactions of trehalose with the common food ingredients, that is, fatty acids, proteins, and ions, presented here may contribute to the further improvement of formulation of biologicals and pharmaceuticals, in particular, to better understand the compatibility of formulation components.

USE IN THE COSMETIC INDUSTRY

Although the exact composition of ingredients in a cosmetic product is typically unknown, the presence of a certain excipient can be determined from product packaging. As is the requirement for every ingredient in a cosmetic product, trehalose has been demonstrated to be a safe and stable natural product that can be used with confidence. Trehalose is used in a wide variety of cosmetic products including bath oils, hair growth tonics, and moisturizers; examples include Lux shampoo (Unilever, Tokyo, Japan), skin milk body lotion (Nivea-Kao, Tokyo, Japan), and Pro Tec style deodorant (Lion, Tokyo, Japan). Trehalose and its sulfate forms are used as moisture-retaining agents in several cosmetic creams and lotions, whereas the fatty acid esters of trehalose are thought to act as surface-active agents.[139] Furthermore, trehalose may be incorporated into cosmetic products to enhance their storage stability and to mask the odor of active ingredient(s) and their degradation products, if any are produced (Table 6). During storage, cosmetics (particularly, cream-based products) may undergo degradation (oxidation) and emit unpleasant odor. As cosmetics are used for skin care, hair care, and makeup, the aroma of these products is a critical factor that can influence its quality and popularity (and thus sales). In one study, the effect of trehalose in suppressing the formation of aldehydes was examined from a variety of oil-based products, including triethylhexanoin, sunflower oil, grape seed oil, and cacao oil.[298] Trehalose was shown to suppress the formation of both short-chain (3–7 carbons) and medium-chain (8–16 carbons) aldehydes and alcohols following storage at 45°C for up to 4 weeks,[298] in most cases by 50% and in some cases by more than 90%.

Table 6. Summary of Protective Effects of Trehalose as a Cosmetic Ingredient

Effects	References
Moisturizer, skin cell preservation	42,303
Stabilizer (i.e., suppression of oil breakdown in cosmetics)	298
Suppression of body odor	24,27,48,49,298
Suppression of free radicals (and their damage to DNA)	27,304

The effectiveness of trehalose in protecting the cell membrane was described previously for liposomal applications. The outer layer of human skin consists of layers of cells that maintain approximately 20% water, which contribute to the texture (i.e., softness) and malleability of the skin, and create a network of cells that acts as a barrier to dehydration and concurrently, intrusion of foreign material.[42] Thus, the maintenance of water content on the outer layer of skin is essential. In a study by Takeuchi and Banno,[42] human skin cells were dried for several hours after reaching confluency, and the effects of several sugars at various concentrations were examined. Trehalose was demonstrated to improve the survival of these cells to desiccation in a concentration-dependent manner (determined by trypan blue staining); in the presence of 0.25% trehalose, the survival rate after 4 h of desiccation increased from 23% (no trehalose) to 41%, and increased further to 45% at 1% concentration.[42] In comparison with trehalose, sucrose was less effective. Thus, trehalose preserved the integrity of cells upon desiccation, as was observed previously for liposomes. For this reason, trehalose may be an effective ingredient in lotions and moisturizers. The low number of commercial products containing trehalose for this purpose may be attributed to its cost, although with the development of novel production methods, this may present less of a hurdle.

Trehalose can also be included in cosmetic products as the key ingredient in suppressing human body odor. Odor associated with aging is caused by the formation of unsaturated aldehydes such as 2-nonenal and 2-octenal. These aldehydes are produced by the degradation of unsaturated fatty acid (palmitoleic acid) in the skin of seniors. In one study, the body of seniors (aged >55 years) was sprayed with 2% trehalose after shower, and 20 h later, the amount of unsaturated aldehydes produced was analyzed from the subjects' shirts. Decrease of about 70% in odor from seniors was noted upon the application of

trehalose solution;[2448] more specifically, the amount of 2-hexenal, 2-octenal, and 2-nonenal production was reduced upon the use of 2% trehalose solution from 17.2 to 5.4 μg, in total.[27] Similar effects were observed with people aged less than 55 years; however, the noted effects were significantly less. Besides the unsaturated aldehydes, free radicals and HPOs are produced during oxidation of fatty acids and may result in more than foul odor. These products can react with proteins and DNA, resulting in scission of DNA chain or its irregular production, potentially leading to deleterious conditions, including cancer.[27] This scenario may be avoided with the use of trehalose, which can suppress the breakdown of fatty acids.

CURRENT REGULATORY ISSUES AND SPECIFICATIONS IN PHARMACOPOEIAS

As described in previous sections, trehalose is a safe ingredient and is utilized in a wide variety of products, including pharmaceuticals, foods, and cosmetics. For its incorporation in commercial products, trehalose must be approved by several governing bodies that determine its safety and efficacy, as intended. Shown below in Table 7 is the regulatory status of trehalose as of June 2010. As a pharmaceutical reagent, trehalose is listed on the United States Pharmacopoeia—National Formulary and European Pharmacopoeia and will be listed on the Japanese Pharmacopoeia in 2011, thus paving the way for its widespread use in the next generation of therapeutic products. The use of trehalose in the food industry has been more prevalent, and is reflected in the earlier date of its approval. In 2000, trehalose was listed as a safe food ingredient by the FDA, and in 2001, as a novel food ingredient in the European Union (EU). The approval in Japan for the use of trehalose as a food ingredient and food additive predates those in the United States and EU by several years (1996) and is reflected in the wide variety of commercial food products that contain trehalose. In addition, trehalose is approved or registered in 21 other countries/regions as a food additive. As an ingredient in cosmetic products, trehalose has been listed on Toxic Substances Control Act (TSCA) in the United States and approved as a nonactive ingredient in Japan. In the EU, trehalose has been preregistered on Registration, Evaluation and Authorization of Chemicals (REACH). Trehalose is also approved or registered in nine other countries/regions for use in cosmetic products. Furthermore, trehalose may be used as a cosmetic ingredient in certain countries (i.e., countries in South East Asia) without approval or registration.

Compared to the history of use of trehalose in food products, its application in pharmaceutical products is still at an infantile stage. However, as more studies are conducted to examine its compatibility with the newly synthesized therapeutic products, the use of trehalose is expected to increase.

FUTURE APPLICATIONS OF TREHALOSE

As described in previous sections, trehalose is a safe ingredient and is utilized in a wide variety of products including pharmaceuticals, foods, and cosmetics. In the pharmaceutical industry, various companies,

Table 7. Summary of Regulatory Status of Trehalose for Pharmaceutical, Food, and Cosmetic use

Region/Agency	Status	Date
Pharmaceutical Use		
USA: United States Pharmacopoeia—National Formulary (USP-NF)	Listed on USP-NF	Jan 2010
EU: European Pharmacopoeia (Ph. Eur.)	Listed on Ph.Eur. 6.8	Jul 2010
Japan: Japanese Pharmacopoeia (JP)	To be listed on the 16th edition of JP	2011
Food Use		
USA	GRAS[a] (FDA notified, No. GRN000045) as food ingredient	Oct 2000
EU	Novel food	Sep 2001
Japan	Listed on LEFA[b], food ingredient, and food additive	1996
JECFA[c]	ADI[d] "not specified"	Jun 2000[e]
Cosmetic Use		
USA	Listed on TSCA[f]	
EU	Preregistered on REACH[g] (EC No. 202-739-6)	
Japan	Nonactive ingredient in approved quasi-drugs	
Chemical Abstracts Service Registry Number (CAS RN.)	6138-23-4 (dihydrate) 99-20-7 (anhydrous)	

[a] GRAS: generally regarded as safe.
[b] LEFA: List of Existing Food Additives.
[c] JECFA: Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Expert Committee on Food Additives.
[d] ADI: Acceptable daily intake.
[e] Issued on June 2000 at the 55th JECFA and published in Food and Nutritional Paper (FNP) 42 Add. 8 (2001).
[f] TSCA: Toxic Substances Control Act.
[g] REACH: Registration, Evaluation, and Authorization of Chemicals, EC: European Commission.

including Biomatrica and Cellphire, are utilizing trehalose as a technology platform based on its stabilizing effect on proteins,[78,232,235,305] platelets,[65,167–170] DNA,[48,259] etc., whereas Genentech possess several patent applications, which describe the use of trehalose as a stabilizer in antibody formulation (Table 2). Other research findings described in the current review, including the beneficial effect of trehalose on osteoporosis[188,189,271] HD,[146,272] dry eye,[179] etc., may lead to other companies adopting trehalose as a therapeutic. Trehalose has been tested clinically for the treatment of dry eye syndrome[179,275,276] and successfully used in organ transplant solution at Kyoto University.[190–195,198] The use of trehalose may be imminent in the solid dosage forms, as evidenced by the high number of patent applications (Table 2). Further understanding of the interaction of trehalose with metal ions, fatty acids, and other small molecules, as described in the section entitled *Use in the Food Industry*, may lead to better formulations through our increased understanding of excipient–excipient interactions. The stability of trehalose to high temperature and hydrolysis may mark the sugar to be a suitable choice for a parenteral formulation as it can be sterilized by autoclave[144] or γ-radiation.[145] In the food and cosmetic industries, the use of trehalose may increase in the United States and Europe following its reduction in cost coupled to its successful and prevalent use in Japan.

Despite the abundance of data demonstrating the benefits of trehalose over some of the currently used stabilizers or food ingredients, acceptance of its use in the various industries and countries may ultimately depend on only a few factors such as cost, familiarity, and intellectual property landscape. The cost hurdle has been reduced significantly due to the recent advancements in trehalose production. However, there still remains the resistance by the user; if the user (or company) is not familiar with the sugar, it will not be adopted. The excipients that trehalsoe may replace, including sucrose, mannitol, lactose, have been utilized in the pharmaceutical industry for a very long time, thus enough incentive must be present for the change to occur. In addition, with further progression of formulation in its development cycle, the incorporation of trehalose (or switch) will be difficult, as is the case with products that are already marketed and being redeveloped as a second-generation product, both from the regulatory perspective. Furthermore, the use of trehalose in certain application or country may be limited by the freedom to operate, as the number of patent applications can attest (Table 2). It is imperative for the reader to be aware of these limitations prior to developing new applications. Nonetheless, we hope that the current review can serve the reader in understanding the unique properties of trehalose, and furthermore, in motivating the reader to apply some of the presented ideas in his/her field of expertise.

SUMMARY

Trehalose, once considered a rare sugar, is prevalent both in nature and in products that are encountered daily, including foods, cosmetics, and pharmaceuticals. The sugar does possess very unique physical and chemical properties, which sets itself apart from the other sugars, and has been the focus of numerous studies. The majority of attention has been placed on its application in preserving biological molecules, including lipids and proteins, and more recently, with stem cells, tissues, and organs. There are, however, a number of studies that examined the effects of trehalose in less well known subjects such as its ability to suppress the formation of foul odor from fish and meat (as well as from humans) and to maintain the appearance and quality of processed food products. Trehalose can be applied to the skin, as a component of a moisturizer, or be eaten, as an ingredient in confectionary products. In both cases, the unique ability of trehalose to bind tightly to water is used to enhance product quality. Drinking trehalose is claimed to ameliorate the conditions of osteoporosis and HD. The use of trehalose in a wide variety of medical applications is being examined and its progress is reflected in the increasing number of patent applications. Of particular interest, in the near future, for the use of trehalose in the pharmaceutical industry is its use as an excipient in solid dosage form. This is due to its numerous advantages over the traditional excipients such as lactose and mannitol. As the number of products introduced in the current review can testify, the use of trehalose is much more prevalent in Japan than in any other country, however, this may soon change. With the development of novel processing methods to improve purity and also affordability, trehalose is certain to find its way into increasing number of products and applications.

ACKNOWLEDGMENTS

The authors would like to thank Dr. George Zografi for valuable comments to this manuscript and Drs. Takanobu Higashiyama and Shigeharu Fukuda for their assistance and support in providing valuable data and references from Hayashibara Biochemical Laboratories.

REFERENCES

1. Furuki T, Oku K, Sakurai M. 2009. Thermodynamic, hydration and structural characteristics of alpha,alpha-trehalose. Front Biosci 14:3523–3535.
2. Jain NK, Roy I. 2009. Effect of trehalose on protein structure. Protein Sci 18(1):24–36.

3. Teramoto N, Sachinvala ND, Shibata M. 2008. Trehalose and trehalose-based polymers for environmentally benign, biocompatible and bioactive materials. Molecules 13(8):1773–1816.
4. Crowe JH. 2007. Trehalose as a "chemical chaperone": Fact and fantasy. Adv Exp Med Biol 594:143–158.
5. Elbein AD, Pan YT, Pastuszak I, Carroll D. 2003. New insights on trehalose: A multifunctional molecule. Glycobiology 13(4):17R–27R.
6. Schiraldi C, Di Lernia I, De Rosa M. 2002. Trehalose production: Exploiting novel approaches. Trends Biotechnol 20(10):420–425.
7. Richards AB, Krakowka S, Dexter LB, Schmid H, Wolterbeek AP, Waalkens-Berendsen DH, Shigoyuki A, Kurimoto M. 2002. Trehalose: A review of properties, history of use and human tolerance, and results of multiple safety studies. Food Chem Toxicol 40(7):871–898.
8. Wingler A. 2002. The function of trehalose biosynthesis in plants. Phytochemistry 60(5):437–440.
9. Crowe JH, Crowe LM, Oliver AE, Tsvetkova N, Wolkers W, Tablin F. 2001. The trehalose myth revisited: Introduction to a symposium on stabilization of cells in the dry state. Cryobiology 43(2):89–105.
10. Arguelles JC. 2000. Physiological roles of trehalose in bacteria and yeasts: A comparative analysis. Arch Microbiol 174(4):217–224.
11. Chen T, Fowler A, Toner M. 2000. Literature review: Supplemented phase diagram of the trehalose–water binary mixture. Cryobiology 40(3):277–282.
12. Crowe JH, Carpenter JF, Crowe LM. 1998. The role of vitrification in anhydrobiosis. Annu Rev Physiol 60:73–103.
13. Behm CA. 1997. The role of trehalose in the physiology of nematodes. Int J Parasitol 27(2):215–229.
14. de-Araujo PS. 1996. The role of trehalose in cell stress. Braz J Med Biol Res 29(7):873–875.
15. Becker A, Schloder P, Steele JE, Wegener G. 1996. The regulation of trehalose metabolism in insects. Experientia 52(5):433–439.
16. Paiva CL, Panek AD. 1996. Biotechnological applications of the disaccharide trehalose. Biotechnol Annu Rev 2:293–314.
17. Panek AD. 1995. Trehalose metabolism—New horizons in technological applications. Braz J Med Biol Res 28(2):169–181.
18. Newman YM, Ring SG, Colaco C. 1993. The role of trehalose and other carbohydrates in biopreservation. Biotechnol Genet Eng Rev 11:263–294.
19. Crowe JH, Hoekstra FA, Crowe LM. 1992. Anhydrobiosis. Annu Rev Physiol 54:579–599.
20. Wiemken A. 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie Van Leeuwenhoek 58(3):209–217.
21. Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ. 1988. Interactions of sugars with membranes. Biochim Biophys Acta 947(2):367–384.
22. Crowe JH, Crowe LM, Carpenter JF, Aurell Wistrom C. 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem J 242(1):1–10.
23. Singer MA, Lindquist S. 1998. Thermotolerance in *Saccharomyces cerevisiae*: The Yin and Yang of trehalose. Trends Biotechnol 16(11):460–468.
24. Higashiyama T. 2002. Novel functions and applications of trehalose. Pure Appl Chem 74(7):1263–1269.
25. Colaco CALS, Roser B. 1995. Trehalose—A multifunctional additive for food preservation. In Food packaging and preservation; Mathlouthi M, Ed. London: Blackie Professional, pp 123–140.
26. Elbein AD. 1974. The metabolism of alpha,alpha-trehalose. Adv Carbohydr Chem Biochem 30:227–256.
27. Kubota M. 2005. New features and properties of trehalose. New Food Industry 47(3):17–29.
28. Saleki-Gerhardt A, Zografi G. 1994. Non–isothermal and isothermal crystallization of sucrose from the amorphous state. Pharm Res 11(8):1166–1173.
29. Sussich F, Urbani R, Princivalle F, Cesaro A. 1998. Polymorphic amorphous and crystalline forms of trehalose. J Am Chem Soc 120:7893–7899.
30. Birch GG. 1963. Trehalose. Adv Carbohydr Chem 18:201–225.
31. Kubota M. 2008. Trehalose-producing enzymes. Fine Chem 37(1):28–35.
32. Roser BJ, Blair J, Colaco C, Hatley RHM. 1996. Oral solid dosage forms, methods of making same and compositions thereof. Patent US 5,958,455.
33. Miller DP, de Pablo JJ, Corti H. 1997. Thermophysical properties of trehalose and its concentrated aqueous solutions. Pharm Res 14(5):578–590.
34. Crowe JH, Hoekstra FA, Nguyen KH, Crowe LM. 1996. Is vitrification involved in depression of the phase transition temperature in dry phospholipids? Biochim Biophys Acta 1280(2):187–196.
35. Crowe LM, Reid DS, Crowe JH. 1996. Is trehalose special for preserving dry biomaterials? Biophys J 71(4):2087–2093.
36. Koster KL, Lei YP, Anderson M, Martin S, Bryant G. 2000. Effects of vitrified and nonvitrified sugars on phosphatidylcholine fluid-to-gel phase transitions. Biophys J 78(4):1932–1946.
37. Roser B. 1991. Trehalose drying: A novel replacement for freeze-drying. BioPharm 5:47–53.
38. Wolfenden R, Yuan Y. 2008. Rates of spontaneous cleavage of glucose, fructose, sucrose, and trehalose in water, and the catalytic proficiencies of invertase and trehalas. J Am Chem Soc 130(24):7548–7549.
39. Karel M, Labuza TP. 1968. Nonenzymatic browning in model systems containing sucrose. J Agric Food Chem 16(5):717–719.
40. Schoebel T, Tannenbaum S, Labuza T. 1969. Reaction at limited water concentration. 1. Sucrose hydrolysis. J Food Sci 34:324.
41. Flink MJ. 1983. Nonenzymatic browning of freeze-dried sucrose. J Food Sci 48:539–542.
42. Takeuchi K, Banno N. 1998. [New applications of trehalose and its potential application in cosmetic products]. Fragrance J 7:39–47.
43. Sola-Penna M, Meyer-Fernandes JR. 1998. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars? Arch Biochem Biophys 360(1):10–14.
44. Tanaka K. 2009. [Development of Treha(R) and its properties]. Food Industry 52(10):45–51.
45. Ekdawi-Sever NC, de Pablo JJ, Feick E, von Meerwall E. 2003. Diffusion of sucrose and α,α-trehalose in aqueous solutions. J Phys Chem A 107:936–943.
46. Ekdawi-Sever NC, Conrad PB, de Pablo JJ. 2001. Molecular simulation of sucrose solutions near the glass transition temperature. J Phys Chem A 105:734–742.
47. Conrad PB, de Pablo JJ. 1999. Computer simulation of the cryoprotectant disaccharide α,α-trehalose in aqueous solution. J Phys Chem A 103:4049–4055.
48. Oku K, Sawatani I, Sugimoto S, Kanbe M, Takeuchi K, Murai S, Kurose M, Kubota M, Fukuda S. 2002. Functional properties of trehalose. J Appl Glycosci 49(3):351–357.
49. Kubota M, Sawatani I, Oku K, Takeuchi K, Murai S. 2004. The developoment of α,α-trehalose production and its applications. J Appl Glycosci 51:63–70.
50. Brown GM, Rohrer DC, Berking B, Beevers CA, Gould RO, Simpson R. 1972. The crystal structure of α,α-trehalose

dihydrate from three independent X-ray determinations. Acta Crystallogr B 28:3145–3158.
51. Brown GM, Levy HA. 1963. Sucrose: Precise determination of crystal and molecular structure by neutron diffraction. Science 141:921–923.
52. Jeffrey GA. 1990. Crystallographic studies of carbohydrates. Acta Crystallogr B 46 (Pt 2):89–103.
53. Jeffrey GA, Saenger W. 1991. Hydration of proteins. In Hydrogen bonding in biological structures; Jeffrey GA, Saenger W, Eds. Berlin: Springer, pp 459–486.
54. Sundaralingam M. 1968. Some aspects of stereochemistry and hydrogen bonding of carbohydrates related to polysaccharide conformations. Biopolymers 6:189–213.
55. Taga T, Senma M, Osaki K. 1972. The crystal and molecular structure of trehalose dihydrate. Acta Crystallogr B 28:3258–3263.
56. Brown GM, Levy HA. 1973. Further refinement of the structure of sucrose based on neutron-diffraction data. Acta Crystallogr B 29:790–797.
57. Jeffrey GA, Sundaralingam M. 1985. Bibliography of crystal structures of carbohydrates, nucleosides, and nucleotides for 1979 and 1980; addenda and errata for 1970–1978; and index for 1935–1980. Adv Carbohydr Chem Biochem 43:203–421.
58. Jeffrey GA, Sundaralingam M. 1976. Bibliography of crystal structures of carbohydrates, nucleosides, and nucleotides 1974. Adv Carbohydr Chem Biochem 32:353–384.
59. Mathlouthi M. 1981. X-Ray diffraction study of the molecular association in aqueous solutions of D-fructose, D-glucose, and sucrose. Carbohydr Res 91:113–123.
60. Kacurakova M, Mathlouthi M. 1996. FTIR and laser-Raman spectra of oligosaccharides in water: Characterization of the glycosidic bond. Carbohydr Res 284(2):145–157.
61. Cohen MH, Grest GS. 1979. Liquid–glass transition, a free-volume approach. Phys Rev B 20:1077–1098.
62. Miller DP, Anderson RE, de Pablo JJ. 1998. Stabilization of lactate dehydrogenase following freeze–thawing and vacuum-drying in the presence of trehalose and borate. Pharm Res 15(8):1215–1221.
63. Mazzobre MF, Del Pilar Buera M. 1999. Combined effects of trehalose and cations on the thermal resistance of beta-galactosidase in freeze-dried systems. Biochim Biophys Acta 1473(2–3):337–344.
64. Ohtake S, Schebor C, Palecek SP, de Pablo JJ. 2004. Effect of sugar–phosphate mixtures on the stability of DPPC membranes in dehydrated systems. Cryobiology 48(1):81–89.
65. Nie Y, de Pablo JJ, Palecek SP. 2005. Platelet cryopreservation using a trehalose and phosphate formulation. Biotechnol Bioeng 92(1):79–90.
66. Sun WQ, Davidson P. 1998. Protein inactivation in amorphous sucrose and trehalose matrices: Effects of phase separation and crystallization. Biochim Biophys Acta 1425(1):235–244.
67. Carpenter JF, Crowe JH. 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28(9):3916–3922.
68. Arakawa T, Kita Y, Carpenter JF. 1991. Protein–solvent interactions in pharmaceutical formulations. Pharm Res 8(3):285–291.
69. Kreilgaard L, Frokjaer S, Flink JM, Randolph TW, Carpenter JF. 1999. Effects of additives on the stability of *Humicola lanuginosa* lipase during freeze-drying and storage in the dried solid. J Pharm Sci 88(3):281–290.
70. Duddu SP, Zhang G, Dal Monte PR. 1997. The relationship between protein aggregation and molecular mobility below the glass transition temperature of lyophilized formulations containing a monoclonal antibody. Pharm Res 14(5):596–600.
71. Jovanovic N, Bouchard A, Hofland GW, Witkamp GJ, Crommelin DJ, Jiskoot W. 2006. Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying. Eur J Pharm Sci 27(4):336–345.
72. Carpenter JF, Crowe LM, Crowe JH. 1987. Stabilization of phosphofructokinase with sugars during freeze-drying: Characterization of enhanced protection in the presence of divalent cations. Biochim Biophys Acta 923(1):109–115.
73. Oku K, Watanabe H, Kubota M, Fukuda S, Kurimoto M, Tsujisaka Y, Komori M, Inoue Y, Sakurai M. 2003. NMR and quantum chemical study on the OH..π and CH..O interactions between trehalose and unsaturated fatty acids: Implication for the mechanism of antioxidant function of trehalose. J Am Chem Soc 125(42):12739–12748.
74. Oku K, Kurose M, Chaen H, Fukuda S, Tsujisaka Y, Sakurai M. 2005. Suppressive effect of trehalose on radical oxidation of unsaturated fatty acids. J Appl Glycosci 52:381–385.
75. Oku K, Chaen H, Fukuda S, Kurimoto M. 1999. [Effect of trehalose on suppression of trimethylamine-formation from boiling fish meat]. Nippon Shokuhin Kagaku Kogaku Kaishi 46(5):319–322.
76. Oku K, Chaen H, Fukuda K, Kurimoto M. 1999. [Suppression of the formation of foul odor by trehalose]. Collection from The Second Trehalose Symposium:65–71.
77. Kurose M. 2006. [Suppression of fish odor by trehalose and its mechanism]. Collection from The Nineth Trehalose Symposium:19–24.
78. Xie G, Timasheff SN. 1997. The thermodynamic mechanism of protein stabilization by trehalose. Biophys Chem 64(1–3):25–43.
79. Kaushik JK, Bhat R. 2003. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. J Biol Chem 278(29):26458–26465.
80. Lin TY, Timasheff SN. 1996. On the role of surface tension in the stabilization of globular proteins. Protein Sci 5(2):372–381.
81. Baptista RP, Pedersen S, Cabrita GJ, Otzen DE, Cabral JM, Melo EP. 2008. Thermodynamics and mechanism of cutinase stabilization by trehalose. Biopolymers 89(6):538–547.
82. Paz-Alfaro KJ, Ruiz-Granados YG, Uribe-Carvajal S, Sampedro JG. 2009. Trehalose-mediated thermal stabilization of glucose oxidase from *Aspergillus niger*. J Biotechnol 141(3–4):130–136.
83. Singer MA, Lindquist S. 1998. Multiple effects of trehalose on protein folding in vitro and in vivo. Mol Cell 1(5):639–648.
84. Ueda T, Nagata M, Imoto T. 2001. Aggregation and chemical reaction in hen lysozyme caused by heating at pH 6 are depressed by osmolytes, sucrose and trehalose. J Biochem 130(4):491–496.
85. Timasheff SN. 1998. Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. Adv Protein Chem 51:355–432.
86. Timasheff SN. 2002. Protein hydration, thermodynamic binding, and preferential hydration. Biochemistry 41(46):13473–13482.
87. Timasheff SN. 2002. Protein–solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. Proc Natl Acad Sci U S A 99(15):9721–9726.
88. Arakawa T, Timasheff SN. 1982. Stabilization of protein structure by sugars. Biochemistry 21(25):6536–6544.
89. Yoshioka S, Miyazaki T, Aso Y, Kawanishi T. 2007. Significance of local mobility in aggregation of beta-galactosidase lyophilized with trehalose, sucrose or stachyose. Pharm Res 24(9):1660–1667.
90. Crowe JH, Crowe LM. 2000. Preservation of mammalian cells—Learning nature's tricks. Nat Biotechnol 18(2):145–146.

91. Franks F. 1990. Freeze drying: From empiricism to predictability. Cryoletters 11:93–110.
92. Crowe LM, Womersley C, Crowe JH, Reid D, Appel L, Rudolph A. 1986. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. Biochim Biophys Acta 861:131–140.
93. Sun WQ, Leopold AC, Crowe LM, Crowe JH. 1996. Stability of dry liposomes in sugar glasses. Biophys J 70(4):1769–1776.
94. Ricker JV, Tsvetkova NM, Wolkers WF, Leidy C, Tablin F, Longo M, Crowe JH. 2003. Trehalose maintains phase separation in an air-dried binary lipid mixture. Biophys J 84(5):3045–3051.
95. Green JL, Angell CA. 1989. Phase relations and vitrification in saccharide–water solutions and the trehalose anomaly. J Phys Chem 93:2880–2882.
96. Roos Y. 1993. Melting and glass transitions of low molecular weight carbohydrates. Carbohydr Res 238:39–48.
97. Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microbiol 61(10):3592–3597.
98. Duddu SP, Dal Monte PR. 1997. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimeric therapeutic monoclonal antibody. Pharm Res 14(5):591–595.
99. Carpenter JF, Crowe JH. 1988. The mechanism of cryoprotection of proteins by solutes. Cryobiology 25(3):244–255.
100. Franks F. Biophysics and biochemistry at low temperatures. Cambridge Univ. Press: Cambridge, 1985.
101. Sundaramurthi P, Suryanarayanan R. 2010. Trehalose crystallization during freeze-drying: Implications on lyoprotection. J Phys Chem Lett 1:510–514.
102. Connolly B, Patapoff TW, Wang YJ, Moore JM, Kamerzell TJ. 2010. Vibrational spectroscopy and chemometrics to characterize and quantitate trehalose crystallization. Anal Biochem 399(1):48–57.
103. Mahdy HM, el-Sheikh HH, Ahmed MS, Refaat BM. 1996. Physiological and biochemical changes induced by osmolality in halotolerant aspergilli. Acta Microbiol Pol 45(1):55–65.
104. Trevelyan WE, Harrison JS. 1956. Studies on yeast metabolism. 5. The trehalose content of Baker's yeast during anaerobic fermentation. Biochem J 62(2):177–183.
105. Nwaka S, Holzer H. 1998. Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. Prog Nucleic Acid Res Mol Biol 58:197–237.
106. Elander M, Myrback K. 1949. Isolation of crystalline trehalose after fermentation of glucose by maceration juice. Arch Biochem 21(2):249–255.
107. Stewart LC, Richtmyer NK, Hudson CS. 1950. Preparation of trehalose from yeast. J Am Chem Soc 72:2059–2061.
108. Wyatt GR, Kale GF. 1957. The chemistry of insect hemolymph. II. Trehalose and other carbohydrates. J Gen Physiol 40(6):833–847.
109. Fairbairn D. 1958. Glucose, trehalose and glycogen in *Porrocaecum decipiens* larvae. Nature 181(4623):1593–1594.
110. Roser B. 1991. Trehalose, a new approach to premium dried foods. Trends Food Sci Tech 2:166–169.
111. Adams RP, Kendall E, Kartha KK. 1990. Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. Biochem Syst Ecol 18:107–110.
112. McBride MJ, Ensign JC 1987. Effects of intracellular trehalose content on *Streptomyces griseus* spores. J Bacteriol 169(11):4995–5001.
113. Van Dijck P, Colavizza D, Smet P, Thevelein JM. 1995. Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 61(1):109–115.
114. Clegg JS. 1965. The origin of trehalose and its significance during the formation of encysted dormant embryos of *Artemia salina*. Comp Biochem Physiol 14:135–143.
115. Womersley C, Smith L. 1981. Anhydrobiosis in nematodes. I. The role of glycerol, myo-inositol and trehalose during dessication. Comp Biochem Physiol 70B:579–586.
116. Gadd GM, Chalmers K, Reed RH. 1987. The role of trehalose in dehydration resistance of *Saccharomyces cerevesiae*. FEMS Microbiol Lett 48:249–254.
117. Asahina E, Tanno K. 1964. A large amount of trehalose in a frost-resistant insect. Nature 204:1222.
118. Sussman AS, Lingappa BT. 1959. role of trehalose in ascospores of *Neurospora tetrasperma*. Science 130(3385):1343.
119. Elbein AD, Mitchell M. 1973. Levels of glycogen and trehalose in *Mycobacterium smegmatis* and the purification and properties of the glycogen synthetase. J Bacteriol 113(2):863–873.
120. Lederer E. 1976. Cord factor and related trehalose esters. Chem Phys Lipids 16(2):91–106.
121. Thevelein JM. 1984. Regulation of trehalose mobilization in fungi. Microbiol Rev 48(1):42–59.
122. Rousseau P, Halvorson HO, Bulla LA, Jr, St Julian G. 1972. Germination and outgrowth of single spores of *Saccharomyces cerevisiae* viewed by scanning electron and phase-contrast microscopy. J Bacteriol 109(3):1232–1238.
123. Attfield PV. 1987. Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response. FEBS Lett 225(1–2):259–263.
124. Cabib E, Leloir LF. 1957. The biosynthesis of trehalose phosphate. J Am Chem Soc 75:259–275.
125. Harding TS. 1923. History of trehalose, its discovery and methods of preparation. Sugar 25:476–478.
126. Sugimoto T. 1995. Production of trehalsoe by enzymatic starch saccharification and its applications. Shokuhin Kogyo (Food Industry) 38:34–39.
127. Candy DJ, Kilby BA. 1958. Site and mode of trehalose biosynthesis in the locust. Nature 183:1584–1595.
128. Murphy TA, Wyatt GR. 1965. The enzymes of glycogen and trehalose synthesis in silk moth fat body. J Biol Chem 240:1500–1508.
129. Lornitzo FA, Goldman DS. 1964. Purification and properties of the transglucosylase inhibitor of *Mycobacterium tuberculosis*. J Biol Chem 239:2730–2734.
130. Roth R, Sussman M. 1966. Trehalose synthesis in the cellular slime mold *Dictyostelium discoideum*. Biochim Biophys Acta 122(2):225–231.
131. Nishimoto T, Nakano M, Bnakada T, Chaen H, Fukuda S, Sugimoto T, Kurimoto M, Tsujisaka Y. 1995. Purification and properties of a novel enzyme, trehalose synthase, from *Pimelobacter* sp. R48. Biosci Biotechnol Biochem 60:640–644.
132. Maruta K, Mitsuzumi H, Nakada T, Kubota M, Chaen H, Fukuda S, Sugimoto T, Kurimoto M. 1996. Cloning and sequencing of a cluster of genes encoding novel enzymes of trehalose biosynthesis from thermophilic archaebacterium *Sulfolobus acidocaldarius*. Biochim Biophys Acta 1291(3):177–181.
133. Steiner A, Cori CF. 1935. The preparation and determination of trehalose in yeast. Science 82(2131):422–423.
134. Leibowitz J. 1944. Isolation of trehalose from desert manna. Biochem J 38(3):205–206.
135. Lemieux RU, Bauer HF. 1953. A chemical synthesis of D-trehalose. Can J Chem 32:340–343.
136. Suzuki T. 1969. The extracellular accumulation of trehalose and glucose by bacteria grown on n-alkanes. Agric Biol Chem 33:190–195.
137. Murao S, Nagano H, Ogura S, Nishino T. 1985. Enzymatic synthesis of trehalose from maltose. Agric Biol Chem 49:2113–2118.

138. Robinson R, Morgan WTJ. 1928. CLIX. Trehalosemonophosphoric ester isolated from the products of fermentation of sugars with dried yeast. Biochem J 22:1277–1288.
139. Kubota M in Glycoenzymes (Ed M. Ohnishi), Japan Scientific Societies Press: Tokyo, 2000, pp 217–225.
140. Nakada T, Maruta K, Mitsuzumi H, Kubota M, Chaen H, Sugimoto T, Kurimoto M, Tsujisaka Y. 1995. Purification and characterization of a novel enzyme, maltooligosyl trehalose trehalohydrolase, from *Arthrobacter* sp. Q36. Biosci Biotechnol Biochem 59(12):2215–2218.
141. Nakada T, Maruta K, Tsusaki K, Kubota M, Chaen H, Sugimoto T, Kurimoto M, Tsujisaka Y. 1995. Purification and properties of a novel enzyme, maltooligosyl trehalose synthase, from *Arthrobacter* sp. Q36. Biosci Biotechnol Biochem 59(12):2210–2214.
142. Maruta K, Nakada T, Kubota M, Chaen H, Sugimoto T, Kurimoto M, Tsujisaka Y. 1995. Formation of trehalose from maltooligosaccharides by a novel enzymatic system. Biosci Biotechnol Biochem 59(10):1829–1834.
143. Yamamoto T, Maruta K, Watanabe H, Yamashita H, Kubota M, Fukuda S, Kurimoto M. 2001. Trehalose-producing operon treYZ from *Arthrobacter ramosus* S34. Biosci Biotechnol Biochem 65(6):1419–1423.
144. Kuniba Y, Arii K, Makita I, Yamashita S. 1992. Nutrient Composition. Patent Japan 06–070718.
145. Caballero I, Castillo A, Naydenov V, Tilquin B. 2005. Radiosensitivity study of glucose and trehalose to gamma irradiation. Am Pharm Rev July/Aug:50–54.
146. Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, Kurosawa M, Nekooki M, Nukina N. 2004. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. Nat Med 10(2):148–154.
147. Yoshioka S, Miyazaki T, Aso Y. 2006. Beta-relaxation of insulin molecule in lyophilized formulations containing trehalose or dextran as a determinant of chemical reactivity. Pharm Res 23(5):961–966.
148. Izutsu K, Yoshioka S, Terao T. 1994. Stabilizing effect of amphiphilic excipients on the freeze–thawing and freeze-drying of lactate dehydrogenase. Biotechnol Bioeng 43(11):1102–1107.
149. Katayama DS, Carpenter JF, Menard KP, Manning MC, Randolph TW. 2009. Mixing properties of lyophilized protein systems: A spectroscopic and calorimetric study. J Pharm Sci 98(9):2954–2969.
150. Garzon-Rodriguez W, Koval RL, Chongprasert S, Krishnan S, Randolph TW, Warne NW, Carpenter JF. 2004. Optimizing storage stability of lyophilized recombinant human interleukin-11 with disaccharide/hydroxyethyl starch mixtures. J Pharm Sci 93(3):684–696.
151. Jameel F, Tchessalov S, Bjornson E, Lu X, Besman M, Pikal M. 2009. Development of freeze-dried biosynthetic Factor VIII: I. A case study in the optimization of formulation. Pharm Dev Technol 14(6):687–697.
152. Abdul-Fattah AM, Lechuga-Ballesteros D, Kalonia DS, Pikal MJ. 2008. The impact of drying method and formulation on the physical properties and stability of methionyl human growth hormone in the amorphous solid state. J Pharm Sci 97(1):163–184.
153. Conrad PB, Miller DP, Cielenski PR, de Pablo JJ. 2000. Stabilization and preservation of *Lactobacillus acidophilus* in saccharide matrices. Cryobiology 41(1):17–24.
154. Prestrelski SJ, Arakawa T, Carpenter JF. 1993. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. Structural studies using infrared spectroscopy. Arch Biochem Biophys 303(2):465–473.
155. Draber P, Draberova E, Novakova M. 1995. Stability of monoclonal IgM antibodies freeze-dried in the presence of trehalose. J Immunol Methods 181(1):37–43.
156. Andya JD, Maa YF, Costantino HR, Nguyen PA, Dasovich N, Sweeney TD, Hsu CC, Shire SJ. 1999. The effect of formulation excipients on protein stability and aerosol performance of spray-dried powders of a recombinant humanized anti-IgE monoclonal antibody. Pharm Res 16(3):350–358.
157. Li S, Patapoff TW, Overcashier D, Hsu C, Nguyen TH, Borchardt RT. 1996. Effects of reducing sugars on the chemical stability of human relaxin in the lyophilized state. J Pharm Sci 85(8):873–877.
158. Borelli MI, Semino MC, Hernandez RE. 1987. Cryopreservation of islets of Langerhans: The use of trehalose as a cryoprotective agent. Med Sci Res 15:299–300.
159. Saha S, Rajamahendran R, Boediono A, Sumantri C, Suzuki T. 1996. Viability of bovine blastocysts obtained after 7, 8 or 9 days of culture in vitro following vitrification and one-step rehydration. Theriogenology 46(2):331–343.
160. Kitahara AK, Suzuki Y, Zhan CW, Wada H, Nishimura Y. 1996. Preservation of skin free-flap using trehalose. J Surg Res 62(1):130–134.
161. Hirata T, Yokomise H, Fukuse T, Muro K, Inui K, Hirai T, Yagi K, Hitomi S, Wada H. 1993. Effects of trehalose in preservation of canine lung for transplantation. J Thorac Cardiovasc Surg 41:59–63.
162. Yokomise H, Inui K, Wada H, Hasegawa S, Ohno N, Hitomi S. 1995. Reliable cryopreservation of trachea for one month in a new trehalose solution. J Thorac Cardiovasc Surg 110(2):382–385.
163. Bhandal IS, Hauptmann RM, Widholm JM. 1985. Trehalose as cryoprotectant for the freeze preservation of carrot and tobacco cells. Plant Physiol 78(2):430–432.
164. McGinnis LK, Zhu L, Lawitts JA, Bhowmick S, Toner M, Biggers JD. 2005. Mouse sperm desiccated and stored in trehalose medium without freezing. Biol Reprod 73(4):627–633.
165. Chen Y, Foote RH, Brockett CC. 1993. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiology 30(4):423–431.
166. Sztein JM, Noble K, Farley JS, Mobraaten LE. 2001. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. Cryobiology 42(1):28–39.
167. Wolkers WF, Walker N, Tamari Y, Tablin F, Crowe JH. 2003. Towards a clinical application of freeze-dried human platelets. Cell Preserv Technol 1:175–188.
168. Wolkers WF, Walker NJ, Tablin F, Crowe JH. 2001. Human platelets loaded with trehalose survive freeze-drying. Cryobiology 42(2):79–87.
169. Pietramaggiori G, Kaipainen A, Ho D, Orser C, Pebley W, Rudolph A, Orgill DP. 2007. Trehalose lyophilized platelets for wound healing. Wound Repair Regen 15(2):213–220.
170. Zhou XL, Zhu H, Zhang SZ, Zhu FM, Chen GM, Yan LX. 2006. Freeze-drying of human platelets: Influence of intracellular trehalose and extracellular protectants. Cryo Lett 27(1):43–50.
171. Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H, Toner M. 2000. Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nat Biotechnol 18(2):163–167.
172. Ohtake S, Martin RA, Yee L, Chen D, Kristensen DD, Lechuga-Ballesteros D, Truong-Le V. 2010. Heat-stable measles vaccine produced by spray drying. Vaccine 28(5):1275–1284.
173. Huang J, Garmise RJ, Crowder TM, Mar K, Hwang CR, Hickey AJ, Mikszta JA, Sullivan VJ. 2004. A novel dry powder influenza vaccine and intranasal delivery technology:

Induction of systemic and mucosal immune responses in rats. Vaccine 23(6):794–801.
174. Adebayo AA, Sim-Brandenburg JW, Emmel H, Olaleye DO, Niedrig M. 1998. Stability of 17D yellow fever virus vaccine using different stabilizers. Biologicals 26(4):309–316.
175. Corbanie EA, Remon JP, Van Reeth K, Landman WJ, van Eck JH, Vervaet C. 2007. Spray drying of an attenuated live Newcastle disease vaccine virus intended for respiratory mass vaccination of poultry. Vaccine 25(49):8306–8317.
176. Worrall EE, Litamoi JK, Seck BM, Ayelet G. 2001. *Xerovac*: An ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines. Vaccine 19:834–839.
177. Ausar SF, Espina M, Brock J, Thyagarayapuran N, Repetto R, Khandke L, Middaugh CR. 2007. High-throughput screening of stabilizers for respiratory syncytial virus: Identification of stabilizers and their effects on the conformational thermostability of viral particles. Hum Vaccin 3(3):94–103.
178. Gribbon EM, Sen SD, Roser BJ, Kampinga J, In New approaches to stabilisation of vaccine potency dev biol stand (Ed. F. Brown), Karger:Basel, 1996, pp 193–199.
179. Matsuo T. 2001. Trehalose protects corneal epithelial cells from death by drying Br J Ophthalmol 85:610–612.
180. Hirata T, Fukuse T, Liu CJ, Muro K, Yokomise H, Yagi K, Inui K, Hitomi S, Wada H. 1994. Effects of trehalose in canine lung preservation. Surgery 115(1):102–107.
181. Neta T, Takada K, Hirasawa M. 2000. Low-cariogenicity of trehalose as a substrate. J Dent 28:571–576.
182. Ukawa Y, Gu Y, Ohtsuki K, Suzuki I, Hisamatsu M. 2005. Antitumor effect of trehalose on Sarcoma 180 in ICR mice. J Appl Glycosci 367–368.
183. Yarkoni E, Lederer E, Rapp HJ. 1981. Immunotherapy of experimental cancer with a mixture of synthetic muramyl dipeptide and trehalose dimycolate. Infect Immun 32(1):273–276.
184. Toubiana R, Ribi E, McLaughlin C, Strain SM. 1977. The effect of synthetic and naturally occurring trehalose fatty acid esters in tumor regression. Cancer Immunol Immunother 2:189–193.
185. Vosika GJ, Schmidtke JR, Goldman A, Ribi E, Parker R, Gray GR. 1979. Intralesional immunotherapy of malignant melanoma with *Mycobacterium smegmatis* cell wall skeleton combined with trehalose dimycolate (P3). Cancer 44(2):495–503.
186. Okabe S, Suganuma M, Tada Y, Ochiai Y, Sueoka E, Kohya H, Shibata A, Takahashi M, Mizutani M, Matsuzaki T, Fujiki H. 1999. Disaccharide esters screened for inhibition of tumor necrosis factor-alpha release are new anti-cancer agents. Jpn J Cancer Res 90(6):669–676.
187. Seyffart G, Rothe W, Bartz V. 1985. Dialysis solution for use in intraperitoneal dialysis. Patent US 4,879,280.
188. Nishizaki Y, Yoshizane C, Toshimori Y, Arai N, Akamatsu S, Hanaya T, Arai S, Ikeda M, Kurimoto M. 2000. Disaccharide-trehalose inhibits bone resorption in ovariectomized mice. Nutr Res 20:653–664.
189. Yoshizane C, Arai N, Arai C, Yamamoto M, Nishizaki Y, Hanaya T, Arai S, Ikeda M, Kurimoto M. 2000. Trehalose suppresses osteoclast differentiation in ovariectomized mice: Correlation with decreased in vitro interleukin-6 production by bone marrow cells. Nutr Res 1485–1491.
190. Omasa M, Hasegawa S, Bando T, Hanaoka N, Yoshimura T, Nakamura T, Wada H. 2004. Application of ET-Kyoto solution in clinical lung transplantation. Ann Thorac Surg 77:338–339.
191. Hirata T, Fukuse T, Ueda Y, Liu CJ, Hitomi S, Wada H. 1996. Effect of new ET-Kyoto solution on 17-hour lung preservation in isolated rat lungs. Transplant Proc 28(3):1922–1923.
192. Liu CJ, Bando T, Hirai T, Hirata T, Yagi K, Yokomise H, Inui K, Hitomi S, Wada H. 1994. Effects of ET-Kyoto solution on 20-hour canine lung preservation. Transplant Proc 26(2):876–877.
193. Fukuse T, Hirata T, Ueda M, Hitomi S, Wada H. 1996. Effects of Euro-Collins, University of Wisconsin, and new extracellular-type trehalase-containing Kyoto solutions in an ex vivo rat lung preservation model. Transplantation 62(9):1212–1217.
194. Bando T, Kosaka S, Liu C, Hirai T, Hirata T, Yokomise H, Yagi K, Inui K, Hitomi S, Wada H. 1994. Effects of newly developed solutions containing trehalose on twenty-hour canine lung preservation. J Thorac Cardiovasc Surg 108(1):92–98.
195. Wada H, Liu CJ, Yokomise H, Inui K, Hirata T, Fukuse T, Hitomi S. 1996. Newly developed preservation solution for the lung: 30-hour preservation with new ET-Kyoto solution. Transplant Proc 28(3):1178–1180.
196. Fukuse T, Hirata T, Nakamura T, Ueda M, Kawashima M, Hitomi S, Wada H. 1999. Role of saccharides on lung preservation. Transplantation 68:110–117.
197. Yokomise H, Inui K, Wada H, Ueda M, Hitomi S. 1996. Long-term cryopreservation can prevent rejection of canine tracheal allografts with preservation of graft viability. J Thorac Cardiovasc Surg 111(5):930–934.
198. Wada H, Fukuse T, Nakamura T, Liu CJ, Bando T, Kosaka S, Ariyasu T, Hitomi S. 1996. ET-Kyoto solution for 48-hour canine lung preservation. Ann Thorac Surg 61(3):963–968.
199. Bando T, Albes JM, Nusse T, Wada H, Hitomi S, Wahlers T, Schafers H. 1998. Comparison of euro-collins solution, low-potassium dextran solution containing glucose, and ET-kyoto solution for lung preservation in an extracorporeal rat lung perfusion model. Eur Surg Res 30(5):297–304.
200. Mizumoto T, Masuda Y, Yamamoto T, Yonemochi E, Terada K. 2005. Formulation design of a novel fast-disintegrating tablet. Int J Pharm 306(1–2):83–90.
201. Mizumoto T, Masuda Y, Kajiyama A, Yanagisawa M, Nyshadham JR. 2004. Quick disintegrating tablet in buccal cavity and production process thereof. Patent Application US10/961,875.
202. Shinoda T, Maeda A, Itou N, Mizumoto T, Yamazaki S, Takaishi Y. 2007. Compositions comprises sustained-release fine particles and manufacturing method thereof. Patent US 7,575,762.
203. Mizumoto T, Masuda Y, Kajiyama A, Yanagisawa M, Nyshadham JR. 2000. Tablets quickly disintegrating in the oral cavity and process for producing the same. Patent US 6,589,554.
204. Roser BJ, Blair J. 1996. Rapidly soluble oral solid dosage forms, methods of making same, and compositions thereof. Patent US 5,762,961.
205. Okada M, Ikeda Y, Ono K, Kurazumi T, Kasai S, Imamori K. 2000. Quickly soluble solid preparations. Patent US 6,455,053.
206. Kajiyama A, Tamura T, Mizumoto T, Kawai H, Takahashi T. 2003 Quick disintegrating tablet in buccal cavity and manufacturing method thereof. Patent US 7,074,428.
207. Yanagisawa M, Mizumoto T. 2002. Bitterness-reduced intrabuccally quick disintegrating tablets and method for reducing bitterness. Patent US 6,998,139.
208. Grimshaw MN, Barbieri DJ, Vizzini LM, Marsh SF. 2007. Rapidly disintegrable tablets. Patent US 7,425,341.
209. Ohkouchi K, Koyama, H. 2001. Quickly disintegrating solid preparations. Patent US 6,740,339.
210. Mizumoto T, Masuda Y, Fukui M. 1995. Intrabuccally dissolving compressed moldings and production process thereof. Patent US 5,576,014.
211. Bollin E Jr, Fletcher MG. 1987. Trehalose as stabilizer and tableting excipient. Patent US 4,762,857.

212. Wang JY, Yang B, Jennings RN, Jr., Protter AA. 1999. Controlled release delivery of peptide or protein. Patent US 6,187,330.
213. Collis MP, Szczepanik SH. 1998. Vehicle for delivery of particles to a sample. Patent US 5,962,310.
214. Patton JS. 1998. Pulmonary delivery of active fragments of parathyroid hormone. Patent US 6,080,721.
215. Cleland JL, Lam X, Kendrick B, Yang J, Yang TH, Overcashier D, Brooks D, Hsu C, Carpenter JF. 2001. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharm Sci 90(3):310–321.
216. Pikal-Cleland KA, Rodriguez-Hornedo N, Amidon GL, Carpenter JF. 2000. Protein denaturation during freezing and thawing in phosphate buffer systems: Monomeric and tetrameric beta-galactosidase. Arch Biochem Biophys 384(2):398–406.
217. Costantino HR, Griebenow K, Mishra P, Langer R, Klibanov AM. 1995. Fourier-transform infrared spectroscopic investigation of protein stability in the lyophilized form. Biochim Biophys Acta 1253(1):69–74.
218. Anchordoquy TJ, Izutsu KI, Randolph TW, Carpenter JF. 2001. Maintenance of quaternary structure in the frozen state stabilizes lactate dehydrogenase during freeze-drying. Arch Biochem Biophys 390(1):35–41.
219. Carpenter JF, Prestrelski SJ, Arakawa T. 1993. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. Arch Biochem Biophys 303(2):456–464.
220. Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. 2001. Factors affecting short-term and long-term stabilities of proteins. Adv Drug Deliv Rev 46(1–3):307–326.
221. Webb SD, Cleland JL, Carpenter JF, Randolph TW. 2003. Effects of annealing lyophilized and spray-lyophilized formulations of recombinant human interferon-gamma. J Pharm Sci 92(4):715–729.
222. Bhatnagar BS, Bogner RH, Pikal MJ. 2007. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol 12(5):505–523.
223. Heller MC, Carpenter JF, Randolph TW. 1996. Effects of phase separating systems on lyophilized hemoglobin. J Pharm Sci 85(12):1358–1362.
224. Chang BS, Kendrick BS, Carpenter JF. 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharm Sci 85(12):1325–1330.
225. Blakeley D, Tolliday B, Colaco C, Roser B. 1990. Dry instant blood typing plate for bedside use. Lancet 336(8719):854–855.
226. Colaco C, Sen S, Thangavelu M, Pinder S, Roser B. 1992. Extraordinary stability of enzymes dried in trehalose: Simplified molecular biology. Biotechnology (NY) 10(9):1007–1011.
227. Uritani M, Takai M, Yoshinaga K. 1995. Protective effect of disaccharides on restriction endonucleases during drying under vacuum. J Biochem 117(4):774–779.
228. Costantino HR, Carrasquillo KG, Cordero RA, Mumenthaler M, Hsu CC, Griebenow K. 1998. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. J Pharm Sci 87(11):1412–1420.
229. Allison SD, Manning MC, Randolph TW, Middleton K, Davis A, Carpenter JF. 2000. Optimization of storage stability of lyophilized actin using combinations of disaccharides and dextran. J Pharm Sci 89(2):199–214.
230. Dong A, Prestrelski SJ, Allison SD, Carpenter JF. 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J Pharm Sci 84(4):415–424.
231. Chi EY, Krishnan S, Randolph TW, Carpenter JF. 2003. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharm Res 20(9):1325–1336.
232. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. 1997. Rational design of stable lyophilized protein formulations: Some practical advice. Pharm Res 14(8):969–975.
233. Carpenter JF, Crowe JH, Arakawa T. 1990. Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. J Dairy Sci 73(12):3627–3636.
234. Carpenter JF, Prestrelski SJ, Dong A. 1998. Application of infrared spectroscopy to development of stable lyophilized protein formulations. Eur J Pharm Biopharm 45(3):231–238.
235. Carpenter JF, Arakawa T, Crowe JH. 1992. Interactions of stabilizing additives with proteins during freeze–thawing and freeze-drying. Dev Biol Stand 74:225–238; discussion 238–229.
236. Kita Y, Arakawa T, Lin TY, Timasheff SN. 1994. Contribution of the surface free energy perturbation to protein–solvent interactions. Biochemistry 33(50):15178–15189.
237. Timasheff SN. 1995. Solvent stabilization of protein structure. Methods Mol Biol 40:253–269.
238. Chang LL, Pikal MJ. 2009. Mechanisms of protein stabilization in the solid state. J Pharm Sci 98(9):2886–2908.
239. Chang LL, Shepherd D, Sun J, Ouellette D, Grant KL, Tang XC, Pikal MJ. 2005. Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? J Pharm Sci 94(7):1427–1444.
240. Luthra SA, Hodge IM, Pikal MJ. 2008. Investigation of the impact of annealing on global molecular mobility in glasses: Optimization for stabilization of amorphous pharmaceuticals. J Pharm Sci 97(9):3865–3882.
241. Shamblin SL, Hancock BC, Pikal MJ. 2006. Coupling between chemical reactivity and structural relaxation in pharmaceutical glasses. Pharm Res 23(10):2254–2268.
242. Tang XC, Pikal MJ. 2005. Measurement of the kinetics of protein unfolding in viscous systems and implications for protein stability in freeze-drying. Pharm Res 22(7):1176–1185.
244. Oliver AE, Crowe LM, Crowe JH. 1998. Methods for dehydration-tolerance: Depression of the phase transition temperature in dry membranes and carbohydrate vitrification. Seed Sci Res 8:211–221.
243. Crowe LM, Crowe JH, Chapman D. 1985. Interaction of carbohydrates with dry dipalmitoylphosphatidylcholine. Arch Biochem Biophys 236(1):289–296.
245. Harrigan PR, Madden TD, Cullis PR. 1990. Protection of liposomes during dehydration or freezing. Chem Phys Lipids 52(2):139–149.
246. Madden TD, Bally MB, Hope MJ, Cullis PR, Schieren HP, Janoff AS. 1985. Protection of large unilamellar vesicles by trehalose during dehydration: Retention of vesicle contents. Biochim Biophys Acta 817(1):67–74.
247. Crowe JH, Crowe LM. 1988. Factors affecting the stability of dry liposomes. Biochim Biophys Acta 939(2):327–334.
248. Ohtake S, Schebor C, Palecek SP, de Pablo JJ. 2005. Phase behavior of freeze-dried phospholipid–cholesterol mixtures stabilized with trehalose. Biochim Biophys Acta 1713(1):57–64.
249. Crowe LM, Crowe JH, Rudolph A, Womersley C, Appel L. 1985. Preservation of freeze-dried liposomes by trehalose. Arch Biochem Biophys 242(1):240–247.
250. Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. 1987. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiology 24(4):324–331.
251. Crowe LM, Crowe JH. 1988. Trehalose and dry dipalmitoylphosphatidylcholine revisited. Biochim Biophys Acta 946(2):193–201.

252. Kawai H, Sakurai M, Inoue Y, Chujo R, Kobayashi S. 1992. Hydration of oligosaccharides: anomalous hydration ability of trehalose. Cryobiology 29(5):599–606.
253. Crowe LM, Crowe JH. 1991. Solution effects on the thermotropic phase transition of unilamellar liposomes. Biochim Biophys Acta 1064(2):267–274.
254. Ohtake S, Schebor C, de Pablo JJ. 2006. Effects of trehalose on the phase behavior of DPPC-cholesterol unilamellar vesicles. Biochim Biophys Acta 1758(1):65–73.
255. Fabrie CH, de Kruijff B, de Gier J. 1990. Protection by sugars against phase transition-induced leak in hydrated dimyristoylphosphatidylcholine liposomes. Biochim Biophys Acta 1024(2):380–384.
256. Crowe JH, Crowe LM, Hoekstra FA. 1989. Phase transitions and permeability changes in dry membranes during rehydration. J Bioenerg Biomembr 21(1):77–91.
257. Crowe JH, Tablin F, Tsvetkova N, Oliver AE, Walker N, Crowe LM. 1999. Are lipid phase transitions responsible for chilling damage in human platelets? Cryobiology 38(3):180–191.
258. Leidy C, Gousset K, Ricker J, Wolkers WF, Tsvetkova NM, Tablin F, Crowe JH. 2004. Lipid phase behavior and stabilization of domains in membranes of platelets. Cell Biochem Biophys 40(2):123–148.
259. Miwa Y. 2007. Possibility of the use of trehalose in the field of alternative medicine. New Food Industry 49(4):20–30.
260. Beattie GM, Crowe JH, Lopez AD, Cirulli V, Ricordi C, Hayek A. 1997. Trehalose: A cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage. Diabetes 46(3):519–523.
261. Guo N, Puhlev I, Brown DR, Mansbridge J, Levine F. 2000. Trehalose expression confers desiccation tolerance on human cells. Nat Biotechnol 18(2):168–171.
262. Reed RH, Borowitzka LJ, Mackay MA, Chudek JA, Foster R, Warr SCR, Moore DJ, Stewart WDP. 1986. Organic solute accumulation in osmotically stressed cyanobacteria. FEMS Microbiol Rev 39:51–56.
263. Mackay MA, Norton RS, Borowitzka LJ. 1984. Organic osmoregulatory solutes in cyanobacteria. J Gen Microbiol 130:2177–2191.
264. Larsen PI, Sydnes LK, Landfald B, Strom AR. 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch Microbiol 147(1):1–7.
265. Galinski E, Truper HG. 1982. Betaine, a compatible solute in the extremely halophilic phototropic bacterium *Ectothiorhodospira halocloris*. FEMS Microbiol Lett 13:357–360.
266. Bonaterra A, Cabrefiga J, Camps J, Montesinos E. 2007. Increasing survival and efficacy of a bacterial biocontrol agent of fire blight of rosaceous plants by means of osmoadaptation. FEMS Microbiol Ecol 61(1):185–195.
267. Zeng Y, Fan H, Chiueh G, Pham B, Martin R, Lechuga-Ballesteros D, Truong VL, Joshi SB, Middaugh CR. 2009. Towards development of stable formulations of a live attenuated bacterial vaccine: A preformulation study facilitated by a biophysical approach. Hum Vaccin 5(5):322–331.
268. Gadducci A, Fanucchi A, Cosio S, Genazzani AR. 1997. Hormone replacement therapy and gynecological cancer. Anticancer Res 17(5B):3793–3798.
269. Kalu DN. 1991. The ovariectomized rat model of postmenopausal bone loss. Bone Miner 15(3):175–191.
270. Vessey MP. 1984. Exogenous hormones in the aetiology of cancer in women. J R Soc Med 77(7):542–549.
271. Arai C, Kohguchi C, Akamatsu S, Arai N, Yoshizane C, Hasegawa N, Hanaya T, Arai S, Ikeda M, Kuromoto M. 2001. Trehalose suppresses lipopolysaccharide-induced osteoclastogenesis bone marrow in mice. Nutr Res 21:993–999.
272. Tanaka M, Machida Y, Nukina N. 2005. A novel therapeutic strategy for polyglutamine diseases by stabilizing aggregation-prone proteins with small molecules. J Mol Med 83(5):343–352.
273. Davies JE, Sarkar S, Rubinsztein DC. 2006. Trehalose reduces aggregate formation and delays pathology in a transgenic mouse model of oculopharyngeal muscular dystrophy. Hum Mol Genet 15(1):23–31.
274. Liu R, Barkhordarian H, Emadi S, Park CB, Sierks MR. 2005. Trehalose differentially inhibits aggregation and neurotoxicity of beta-amyloid 40 and 42. Neurobiol Dis 20(1):74–81.
275. Matsuo T, Tsuchida Y, Morimoto N. 2002. Trehalose eye drops in the treatment of dry eye syndrome. Ophthalmology 109(11):2024–2029.
276. Matsuo T. 2004. Trehalose versus hyaluronan or cellulose in eye drops for the treatment of dry eye. Jpn J Ophthalmol 48:321–327.
277. Kuno Y, Kojima M, Ando S, Nakagami H. 2008. Effect of preparation method on properties of orally disintegrating tablets made by phase transition. Int J Pharm 355(1–2):87–92.
278. Chakravarty P, Bhardwaj SP, King L, Suryanarayanan R. 2009. Monitoring phase transformations in intact tablets of trehalose by FT-Raman spectroscopy. AAPS PharmSciTech 10(4):1420–1426.
279. Portmann MO, Birch GG. 1995. Sweet taste and solution properties of α,α-trehalose. J Sci Food Agric 69:275–281.
280. Richards AB, Dexter LB, in *Alternative Sweeteners* (Ed.L.O. Nabors), Marcel Dekker, Inc.: New YorkNY. 2001, pp 423–461.
281. Lee CK, Birch GG. 1975. Structural functions of taste in the sugar series: Binding characteristics of disaccharides. J Sci Food Agri 26:1513–1521.
282. Birch GG. 1970. Mushroom sugar in food technology. Process Biochemistry 5: 9.
283. Oku K, Sawatani I, Chaen H, Fukuda S, Kurimoto M. 1998. Trehalose Content in Foods. Nippon Shokuhin Kagaku Kogaku Kaishi 45(6):381–384.
284. Oda Y, Uno K, Ohta S. 1986. Selection of yeasts for bread-making by the frozen-dough method. Appl Environ Microbiol 52:941–943.
285. Patel GB, Ingledew WM. 1973. Internal carbohydrates of *Saccharomyces carlsbergensis* during commercial lager brewing. J Inst Brew 79:392–396.
286. Bertrand A, Dubernet MO, Ribereau Gayon P. 1975. [Trehalose, the principle disaccharide of wine]. CR Hebd Seances Acad Sci D 280:1907–1910.
287. Olano A, Gomez Cordoves MC. 1985. Anal Bromatol 35:171–176.
288. Bogdanov S, Baumann E. 1988. Bestimmung von Honigzucker mit HPLC. Mitt Geb Lebensmittelunters Hyg 79:198–206.
289. Mikolajczak KL, Smith CR, Jr., Wolff IA. 1970. Phenolic and sugar components of Armavirec variety sunflower (*Helianthus annuus*) seed meal. J Agric Food Chem 18:27–32.
290. Dahlqvist A. 1962. Specificity of the human intestinal disaccharidases and implications for hereditary disaccharide intolerance. J Clin Invest 41:463–470.
291. Welsh JD, Poley JR, Bhatia M, Stevenson DE. 1978. Intestinal disaccharidase activities in relation to age, race, and mucosal damage. Gastroenterology 75(5):847–855.
292. Ushijima T, Fugisawa T, Kretchmer N. 1995. [Evaluation of the ability of human small intestine to adsorb trehalose]. Shoka to Kushu (Digestion and Absorption) 18:56–57.
293. Bergoz R. 1971. Trehalose malabsorption causing intolerance to mushrooms. Report of a probable case. Gastroenterology 60(5):909–912.

294. Bolte JP, Schonhage F, Forster E, Knolle J, Meyer KH. 1973. [Diagnostic significance of the trehalose tolerance test in malabsorption syndromes]. Dtsch Med Wochenschr 98(28):1358–1362.
295. Oku T, Okazaki M. 1998. Transitory laxative threshold of trehalose and lactulose in healthy women. J Nutr Sci Vitaminol (Tokyo) 44(6):787–798.
296. Aga H, Shibuya T, Chaen H, Fukuda S, Kurimoto M. 1998. [Stabilization by trehalose of superoxide dismutase-like activity of various vegetables]. Nippon Shokuhin Kagaku Kogaku Kaishi 45(3):210–215.
298. Yamashita H. 2009. [Inhibitive effect of trehalose on odor formation and its application to cosmetics]. Skin Beauty 41(3):146–151.
299. Himei S. 2008. [New developments in the use of Treha(R) in the food industry]. Food Chem 7:25–29.
297. Schebor C, Burin L, Buera MP, Chirife J. 1999. Stability to hydrolysis and browning of trehalose, sucrose and raffinose in low-moisture systems in relation to their use as protectants of dry biomaterials. Lebensmittel-Wissenschaft und-Technologie 32:481–485.
300. Takeuchi K, Banno N. 2000. [Function and application of trehalose (5): Application of cosmetical and pharmaceutical field]. Fragrance J 5:101–103.
301. Oku K, Okazaki M. 1998. [Allowable amount and metabolism of trehalose in Japanese population]. Collection from The First Trehalsoe Symposium:16–30.
302. Muraoka I. 1999. [The potential of trehalose use in sports]. Collection from The Second Trehalose Symposium:49–58.
303. Kojima H. 1999. [Moisturizing of Skin and Trehalose]. Collection from The Second Trehalose Symposium:59–64.
304. Kubota M. 2000. [Prevention of Rotten Odor of Fatty Acids by Trehalose]. Collection from The Third Trehalose Symposium:30–35.
305. Chang LL, Shepherd D, Sun J, Ouellette D, Grant KL, Tang XC, Pikal MJ. 2005. Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? J Pharm Sci 94(7):1427–1444.