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Efficacy of sugar alcohols and sugars in protein stabilization during freezing, freeze-drying, and air-drying

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Abstract

Objectives: Cold-acclimated organisms accumulate low molecular weight organic solutes such as sugar alcohols and soluble sugars. This study aimed to compare the efficacy of five sugar alcohols and 14 soluble sugars in stabilizing proteins under freezing, freeze-drying, and air-drying stresses. **Materials and methods:** Glucose-6-Phosphate Dehydrogenase (G6PD) was used as the model protein. G6PD solutions with or without sugar alcohols and or sugars were subjected to freezing, freeze-drying, and air-drying stresses. The recovery of G6PD activity was measured to evaluate the protective efficacy of these compounds. **Results:** Without stabilizers, freezing G6PD at -20°C or -80°C reduced enzyme activity by around 24%, while freeze-drying or air-drying reduced activity by 90%-95%. Among the five sugar alcohols tested, pinitol, quebrachitol and sorbitol stabilized G6PD, whereas mannitol and myo-inositol destabilized it. Among 14 soluble sugars, trehalose and raffinose showed slightly lower enzyme recovery after repeated freeze-thaw cycles at -20°C. Most soluble sugars (except arabinose and xylose) protected G6PD during freeze-drying, with di-, tri-, and oligosaccharides generally outperforming monosaccharides. During air-drying, lactose was ineffective, while arabinose, galactose, and xylose were detrimental. **Conclusion:** The study highlights the diverse mechanisms of sugar alcohols and sugars in protein stabilization under stress, offering insights for formulating stable protein- and cell-based drugs.

Keywords

desiccation tolerance; freezing tolerance; protein stabilization; sugar alcohols; sugars

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1 Introduction

Protein denaturation and degradation can occur in solution, as well as in the frozen or dehydrated state. Several classes of low molecular weight organic solutes, known as compatible solutes, are recognized for their ability to stabilize labile proteins under freezing and desiccation stresses. Cold-acclimated organisms accumulate large quantities of such compatible solutes, including sugar alcohols and soluble sugars (*i.e.*, mono-, di-, and oligosaccharides). The protective effects of sugar alcohols and soluble sugars on proteins against cold denaturation and dehydration have been well documented[1-4].

The relative efficacy of various sugar alcohols and sugars in protein stabilization during freezing, freeze-drying, or air-drying has also been reported[1-8]. However, most studies have focused on only three sugar alcohols (mannitol, myo-inositol, and sorbitol) and four sugars (glucose, lactose, sucrose, and trehalose), primarily using a limited number of labile enzymes (*e.g.*, alkaline phosphatase, lactate dehydrogenase, phosphofructokinase, restriction endonuclease, and ribonuclease). In contrast, a much broader range of sugar alcohols and sugars accumulate in the cells and tissues of stress-resistant organisms in response to cold, freezing, drought, radiation, and salinity. For example, desiccation-tolerant plant seeds contain large quantities of cyclitols and raffinose-family oligosaccharides[9-11]. In some organisms, cyclitols appear to be preferred over acyclic polyols such as sorbitol and mannitol[12-15]. Nature has evolved highly sophisticated mechanisms to adapt to various environmental stresses.

This study compared the efficacy of five sugar alcohols and 14 soluble sugars in stabilizing Glucose-6-Phosphate Dehydrogenase (G6PD) during freezing, freeze-drying, and air-drying. The goal was to identify natural protective mechanisms that could provide valuable insights for developing formulations of shelf-stable protein- and cell-based drugs.

2 Materials and Methods

2.1 Chemical reagents

G6PD (EC 1.1.1.49) and various sugars and sugar alcohols were purchased from Sigma-Aldrich Chemical Ltd. (St. Louis, MO, USA), including 14 sugars and 5 sugar alcohols. The sugars included D-(-)-arabinose, D-(-)-fructose, D-(+)-galactose, α-D-(+)-glucose, β-D-(+)-glucose, α-D-lactose, maltose, D-(+)-mannose, D-(+)-melezitose, D-(+)-raffinose, sucrose, stachyose, D-(+)-trehalose, and D-(+)-xylose. The sugar alcohols included D-mannitol, D-sorbitol, myo-inositol, D-pinitol, and L-quebrachitol. For comparison, Fig. 1 shows the molecular structures of these sugar alcohols.

2.2 Sample preparation

G6PD was delivered as a suspension in 50 mmol/L Tris-HCl buffer (pH 7.5) containing 3.2 mol/L ammonia sulfate and 1 mmol/L magnesium chloride. The enzyme was desalted before use by passing it through a 10-DG desalting column (cut-off, 4 kD) (Bio-Rad, Hercules, CA, USA). Stock solutions of sugar alcohols and soluble sugars were prepared at 20% (w/w) in 50 mmol/L Tris-HCl buffer (pH 7.5). G6PD solutions, with or without sugar alcohols and/or soluble sugars, were prepared by mixing the desalted G6PD with carbohydrate stock solutions to achieve a final concentration of 10% (w/v). Aliquots (15 L) of enzyme/carbohydrate solutions were pipetted into 1.5-mL Eppendorf tubes.

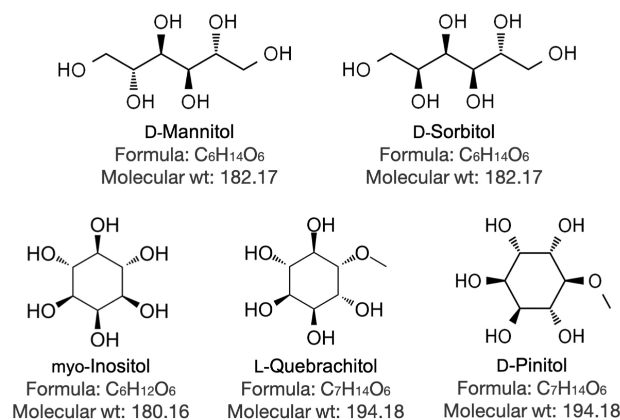

Fig. 1 Chemical structures of five sugar alcohols that used in the protein stabilization study

2.3 Freezing and drying treatment

To evaluate enzyme protection against freezing damage, enzyme samples were frozen at either -20°C or -80°C for 24 hours. For the freezing experiment at -20°C, five freeze-thaw cycles were performed. To assess enzyme protection during freeze-drying, enzyme samples were pre-frozen at -80°C and then lyophilized for 24 hours. To evaluate enzyme protection during air-drying, enzyme samples were dried in a vacuum desiccator containing phosphorus pentoxide (around 1% relative humidity) at around 28°C for 72 hours. The air-drying condition represented a form of flash drying. The residual moisture content of the dried samples was less than 3% for both freeze-drying and air-drying. At least six replicates were used for each measurement.

2.4 Enzyme activity measurement

Enzyme activities of the samples were measured after freezing, freeze-drying, or air-drying, and the percent recovery of enzyme activity was used to assess the efficacy of protein protection by various sugar alcohols and soluble sugars. G6PD activity was measured using the standard method described by Deutsch[7]. For each sample tube, 1.0 mL of working reagent was added, containing 50 mmol/L Tris-HCl buffer (pH 7.5), 6.3 mmol/L magnesium chloride, 3.3 mmol/L glucose-6-phosphate, and 0.38 mmol/L Nicotinamide Adenine Dinucleotide Phosphate (NADP). The mixture was thoroughly mixed for five seconds, and the change in absorbance at 339 nm over time (*i.e.*, the slope) was used to calculate enzyme activity.

3 Results

3.1 G6PD stabilization after freeze/thaw cycles

G6PD stabilization by sugar alcohols and soluble sugars was evaluated after five freeze/thaw cycles at -20°C (Fig. 2, red bars). G6PD was stable in Tris-HCl buffer (pH 7.5) without any stabilizer, retaining about 76% of its enzyme activity after five freeze/thaw cycles.

Among the five sugar alcohols, mannitol and myo-inositol significantly decreased enzyme activity after freezing and thawing at -20°C. Among the 14 tested sugars, trehalose and raffinose showed lower enzyme activity recovery than the buffer-alone control group. Other sugar alcohols and sugars tested did not significantly affect enzyme activity (77%-82%) upon freeze/thaw treatment.

The findings were further verified upon freezing at -80°C (Fig. 2, blue bars), where mannitol and myo-inositol again significantly decreased enzyme activity.

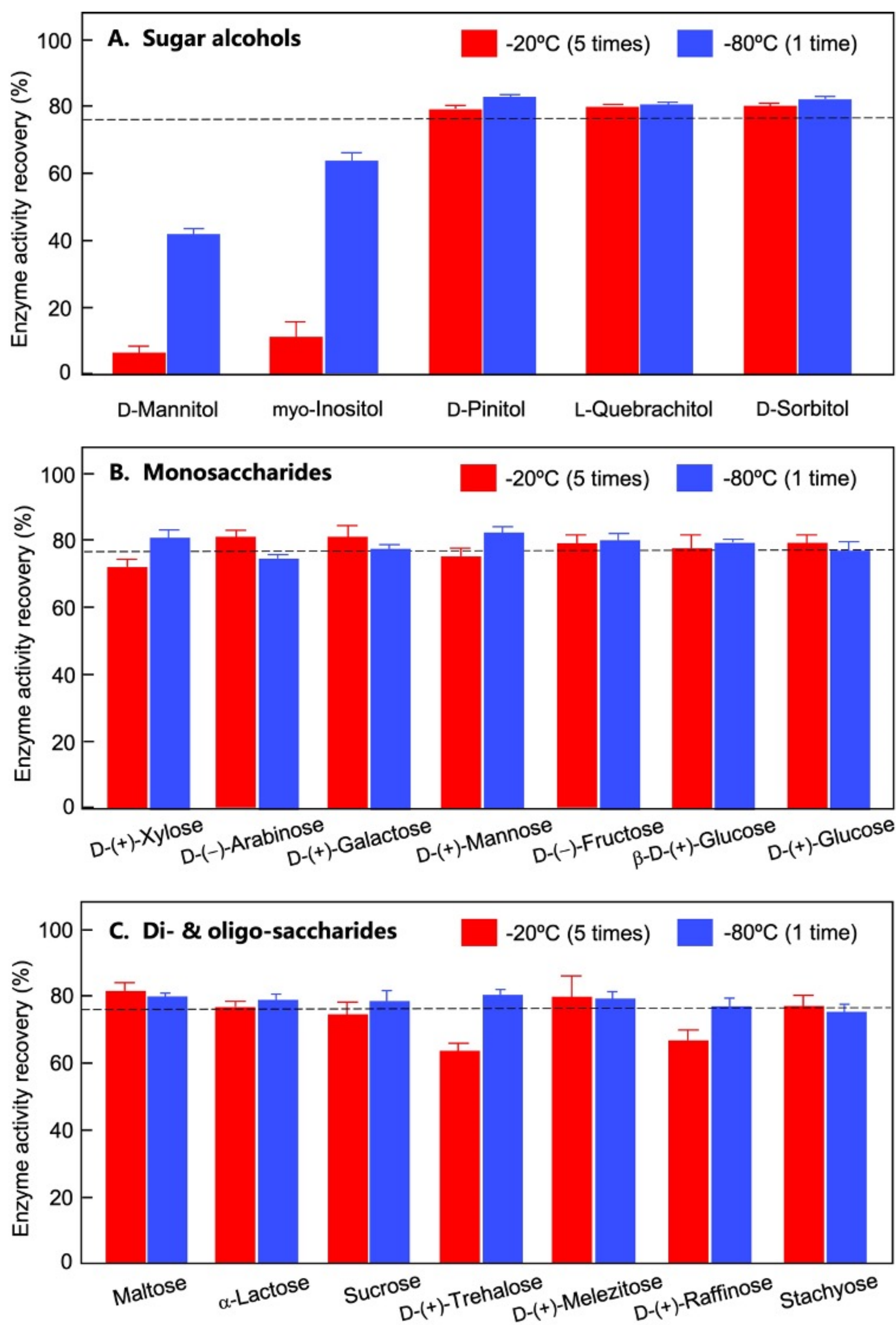

Fig. 2 Recovered activity of glucose-6-phosphate dehydrogenase (in 50 mmol/L Tris-HCl buffer , pH 7.5) after five freeze/thaw cycles at -20°C or one freeze/thaw cycle at -80°C in the presence of sugar alcohols and sugars

The recovery of enzyme activity was 76% in the absence of a protectant (the dashed line). Data are means SE of at least six replicate.

3.2 G6PD stabilization after freeze-drying

Freeze-drying imposed stresses from both freezing and dehydration. Without any stabilizer, G6PD activity was reduced by 96% after freeze-drying (Fig. 3, red bars).

Among the five sugar alcohols, mannitol and myo-inositol were ineffective, yielding lower enzyme activity recovery than the buffer-alone control group (Fig. 3A). Only pinitol, quebrachitol, and sorbitol protected G6PD during freeze-drying, with enzyme activity recovery levels of 81%, 75%, and 66%, respectively (Fig. 3A).

Among the seven monosaccharides, xylose and arabinose were ineffective, whereas galactose, mannose, fructose, and glucose provided varying degrees of protection (Fig. 3B). Glucose yielded the highest recovery (53%), followed by fructose (43%), mannose (36%), and galactose (22%).

Four disaccharides (maltose, lactose, sucrose, and trehalose) and three oligosaccharides (melezitose, raffinose, and stachyose) were all effective protectants (75-83%) for freeze-drying (Fig. 3C), without any additional significant enzyme activity loss compared to freezing at -80°C (Fig. 2C). Among the oligosaccharides, melezitose showed the highest enzyme activity recovery (83%). Among the disaccharides, trehalose and sucrose provided the best enzyme activity recovery (around 80%), followed by lactose (76%) and maltose (72%).

The overall order of efficacy in protein stabilization by sugar alcohols and sugars for freeze-drying was: Melezitose/Pinitol > Raffinose/Trehalose/Sucrose > Stachyose/Lactose > Maltose/Quebrachitol/Sorbitol > Glucose > Fructose > Mannose > Galactose.

3.3 G6PD stabilization after air-drying

G6PD stabilization by sugar alcohols and soluble sugars against dehydration stress was also evaluated by air-drying at room temperature (around 28°C) under low relative humidity (around 1%). In the absence of any stabilizer, enzyme activity decreased by 90% after air-drying.

In general, air-drying resulted in a greater loss of enzyme activity than freeze-drying for enzyme preparations containing sugar alcohols and monosaccharides (Fig. 3A and Fig. 3B). The enzyme activity recovery in the presence of mannitol and myo-inositol was again lower than that of the buffer-alone control group (Fig. 3A). However, pinitol, quebrachitol, and sorbitol provided protective effects during air-drying. Among the sugar alcohols, sorbitol yielded the highest enzyme activity recovery (59%) after air-drying, followed by pinitol (35%) and quebrachitol (29%).

A large variation in enzyme activity recovery was observed in air-dried pinitol samples, ranging from 16% to 65%. Visual observation suggested that D-pinitol provided good enzyme stabilization when the dried sample remained in an amorphous state. In contrast, crystallization of the pinitol sample was associated with low enzyme activity recovery after air-drying.

Among the monosaccharides, fructose yielded the highest recovery (around 42%), followed by glucose (31%) and mannose (22%). In contrast, xylose, arabinose, and galactose appeared to be detrimental, with enzyme activity recovery lower than that of the buffer-alone control group.

Among the disaccharides and oligosaccharides, sucrose and melezitose provided the highest enzyme activity recovery (around 79%), followed by trehalose, raffinose, and stachyose (around 75%) and maltose (around 52%). Lactose did not protect the enzyme during air-drying.

The overall order of efficacy in enzyme stabilization by sugar alcohols and sugars during air-drying was: Sucrose/Melezitose > Trehalose/Raffinose/Stachyose > Sorbitol > Maltose/Fructose > Glucose/Pinitol > Mannose/Quebrachitol.

4 Discussion

The findings of the present study on the efficacy of sugar alcohols for protein protection are consistent with the observation that the adaptive accumulation of sorbitol occurs widely in desiccation-tolerant and freeze-tolerant organisms. In contrast, the accumulation of other sugar alcohols occurs mainly in response to less severe osmotic or chilling stress (as illustrated in Table 1). Mannitol does not accumulate in organisms under freezing conditions. Although myo-inositol is reported to accumulate in some freeze-resistant organisms, its concentration remains relatively low compared to other carbohydrates.

The role of pinitol and other cyclitols in stress resistance in plants

Table 1 The adaptive accumulation of sugar alcohols in stress-tolerant organisms in response to freezing (< 0°C), chilling (> 0°C), salinity and desiccation

Sugar alcohol	Stress Factor			
	Freezing	Chilling	Salinity	Desiccation
Mannitol	ns	+	+ + +	+ + +
Sorbitol	+ + +	+ + +	+ + +	+ + +
Myo-Inositol	+	+ +	+ +	+ +
Ononitol	ns	ns	+ + +	+ + +
Quebrachitol	ns	ns	ns	ns
Pinitol	+ +	+ +	+ + +	+ + +

+: The adaptive accumulation of such a solute was reported in some organisms; + +: The adaptive accumulation of such a solute is observed in a number of cases; + + +: The adaptive accumulation of such a solute has been widely observed; ns: No data was reported in the subject.

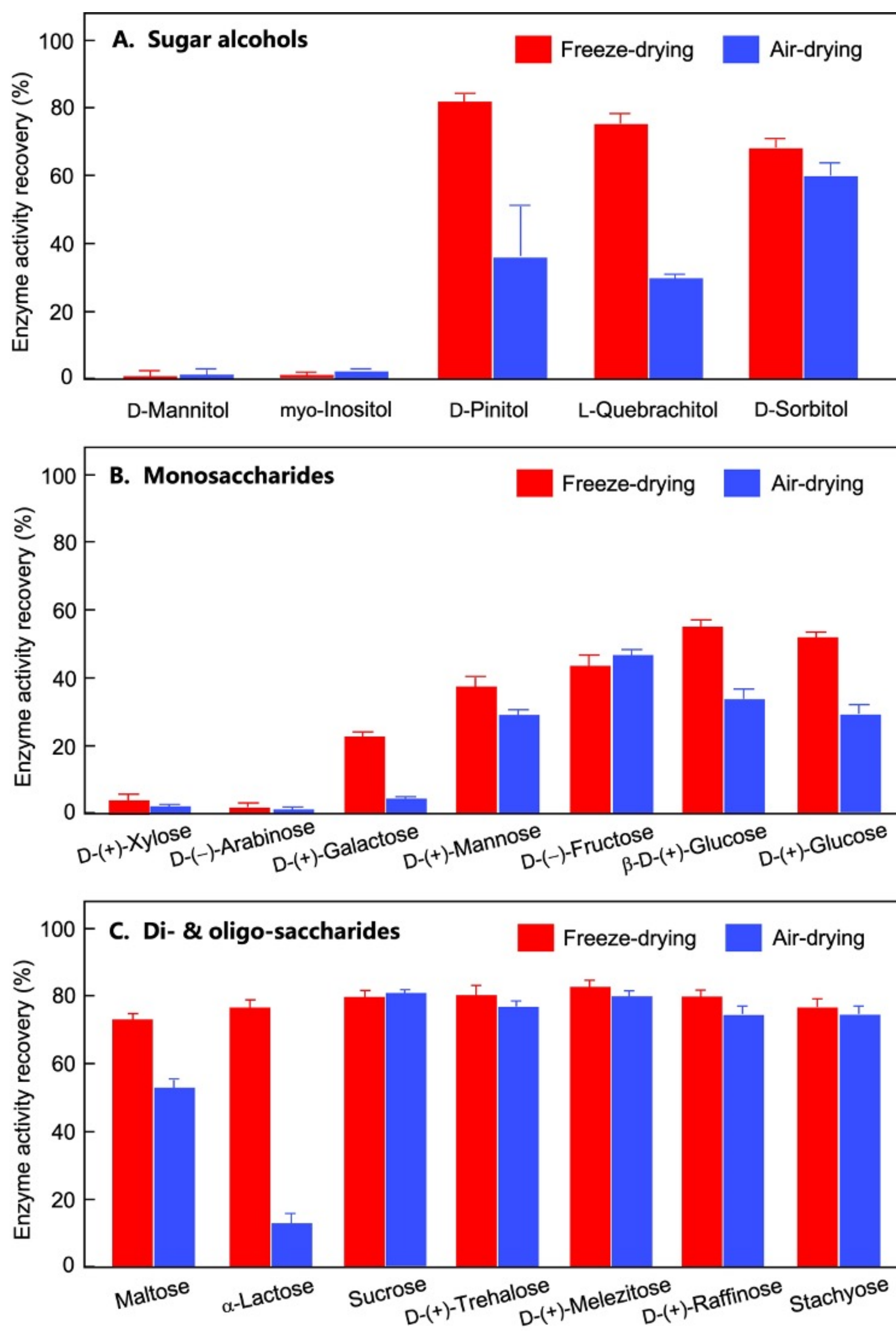

Fig. 3 Recovered activity of glucose-6-phosphate dehydrogenase (in 50 mmol/L Tris-HCl buffer , pH 7.5) after freeze-drying or air-drying in the presence of sugar alcohols and sugars

In the absence of a protectant, the recovery of enzyme activity was 4% and 10% after freeze-drying and air-drying respectively. Data are means SE of at least six replicates.

and protein stability has been reported[12-15]. Pinitol accumulates in tissues under various stress conditions, including chilling, freezing, desiccation, and salinity, and may account for more than 50% of the total soluble carbohydrate pool[12]. Quebrachitol is a versatile building block for naturally occurring bioactive materials and has been reported to improve the thermal stability of proteins, despite the fact that its protective mechanisms remain elusive[15,17]. Cyclitols can function as antioxidants, and protein stabilization by cyclitols during freezing and drying is likely related to their unique structural properties (Fig. 1).

Trehalose is one of the most effective cryoprotectants for protein stabilization upon freezing[1-3,6-8]. Therefore, it was unexpected that the presence of trehalose and raffinose reduced enzyme activity recovery after repeated freeze/thaw cycles at -20°C (Fig. 2). No similar results have been reported in previous studies using lactate dehydrogenase, phosphofructokinase, or other labile enzymes. The mechanism underlying this damage remains unknown. However, it is noted that during freezing, trehalose and raffinose may crystallize as their respective hydrates[19-21]. Further investigation is needed to determine whether the crystallization of trehalose and raffinose is related to the low recovery of enzyme activity after repeated freeze/thaw cycles. Enzyme inactivation in dried sugar matrices has been reported when one component crystallized[10].

Freezing and drying are distinct stress conditions, requiring different mechanisms to stabilize enzymes[21]. The specificity of sugars for protein stabilization during freezing was low, as shown in Fig. 2. However, not all sugars protected the enzyme during freeze-drying and air-drying. Disaccharides, trisaccharides, and oligosaccharides provided excellent enzyme protection during freeze-drying, followed by monosaccharides (Fig. 3).

The efficacy order for protein protection during freeze-drying was: Melezitose > Raffinose/Trehalose/Sucrose > Stachyose/Lactose > Maltose > Glucose > Fructose > Mannose > Galactose.

The efficacy order for protein protection during air-drying was: Sucrose/Melezitose > Trehalose/Raffinose/Stachyose > Maltose/Fructose > Glucose > Mannose.

For membrane protection, the efficacy order of sugars was previously reported as Trehalose > Maltose > Sucrose > Glucose[22]. An attempt to rationalize this efficacy order found that it corresponded exactly with the order of glass transition temperatures (Tg)[23]. However, a similar Tg-based rationale for protein stabilization during drying was not supported by the present study. According to the Tg order of sugars, the expected efficacy order in protein stabilization would be: Trehalose > Stachyose > Raffinose > Lactose > Maltose > Sucrose > Glucose > Galactose / Mannose > Xylose > Fructose.

However, this expected order was not observed in this study. For example, sucrose, if not superior, was at least as effective as trehalose. The present study highlights the diverse mechanisms of natural adaptation by sugar alcohols and sugars in protein stabilization under freezing and dehydration stresses in biological systems. The differential accumulation of sugar alcohols and sugars in organisms likely reflects their varying efficacy in protecting biological systems under different stress conditions. This provides a valuable lesson from nature.

Several mechanisms have been proposed to explain the stabilization effects of compatible low-molecular-weight organic solutes on proteins. These include the colligative action of solutes, specific interactions with proteins, and preferential hydration in unfrozen solutions. To date, only the preferential exclusion theory, established by Timasheff and his co-workers[24-26], provides a satisfactory explanation for the nonspecific stabilization effect of many solutes on proteins. However, the data on the five sugar alcohols presented in this study cannot be fully explained by the preferential exclusion theory.

Mannitol, myo-inositol, and sorbitol are known to be preferentially excluded from the surface of proteins in unfrozen solutions. Yet, mannitol and myo-inositol were detrimental to G6PD during freeze/thaw cycles. While the eutectic separation of mannitol may contribute to enzyme destabilization[27], the destabilizing effect of myo-inositol cannot be explained in the same manner. The extent of freeze-induced concentration also does not account for the observed results. The unfrozen water content in maximally freeze-concentrated myo-inositol solution was reported to be 0.30 g/g. Sorbitol and several sugars tested in this study (Fig. 2) have even lower unfrozen water content than myo-inositol[28]. In previous studies using labile enzymes, the detrimental effects of mannitol and myo-inositol were not observed because the recovery of enzyme activity in control samples was already too low (close to 0%) after freezing, making it impossible to detect additional negative effects of solutes.

Several other reported cases also demonstrate that the preferential exclusion theory does not fully explain the observed effects of certain solutes on protein stabilization under freeze/thaw conditions. It has been observed that the same solute can act as either a cryoprotectant or a cryo-sensitizer, depending on concentration and freezing conditions. A detrimental effect has been reported for ethylene glycol, DMSO, glycerol, myo-inositol, and other solutes at low concentrations[2,29-32]. Myo-inositol provided no stabilization to lactate dehydrogenase during freeze/thaw treatment except at concentrations above 0.6 mol/L; at lower concentrations (below 0.5 mol/L), it appeared to destabilize the enzyme[2]. In this study,

the concentrations of mannitol and myo-inositol were 0.55 mol/L. Even sucrose, at low concentrations, has been shown to have a harmful effect on α-amylase under freezing conditions[32]. These concentration-dependent stabilization effects are not consistent with the preferential exclusion theory[29-31], suggesting that other mechanisms are involved.

In pharmaceutical formulations, mannitol is commonly used as a bulking agent to prevent protein loss during freeze-drying and to improve the appearance of dried products. However, mannitol is not an effective protein stabilizer during freeze-drying and tends to undergo phase separation by crystallization during freezing[19]. The recovery of protein activity after freezing and drying is typically low when mannitol is used alone, without an additional protein stabilizer. Given that a good excipient for protein protection during freezing and drying must function as both a protein structure stabilizer and a good glass former, pinitol, quebrachitol, and sorbitol may be superior alternatives to mannitol for the formulation of protein-based pharmaceutical products. This finding provides valuable insights for developing stable, shelf-ready protein- and cell-based drugs.

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Research ethics

Not applicable.

Informed consent

Not applicable.

Author contributions

Sun W Q conceptualized and designed the study; Sun W Q and Luo Y Q performed experiments and analyzed the data; Sun W Q wrote the article.

Use of large language models, AI and machine learning tools

No LLM, AI or machine learning tool was used for any part of the present study.

Conflict of interest

The author declares no competing interest with respect to the research, authorship, and publication of this article.

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Data availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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