HHS Public Access

Author manuscript

Biochemistry. Author manuscript; available in PMC 2019 October 05.

Published in final edited form as:

Biochemistry. 2019 September 17; 58(37): 3825–3833. doi:10.1021/acs.biochem.9b00675.

Protecting Enzymes from Stress-Induced Inactivation

Samantha Piszkiewicz[†], **Gary J. Pielak**[*,†,‡,§,‖]

[†]Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599, United States

[‡]Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599, United States

[§]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599, United States

[‖]Integrative Program for Biological and Genome Sciences, University of North Carolina, Chapel Hill, North Carolina 27599, United States

Abstract

The pharmaceutical and chemical industries depend on additives to protect enzymes and other proteins against stresses that accompany their manufacture, transport, and storage. Common stresses include vacuum-drying, freeze-thawing, and freeze-drying. The additives include sugars, compatible osmolytes, amino acids, synthetic polymers, and both globular and disordered proteins. Scores of studies have been published on protection, but the data have never been analyzed systematically. To spur efforts to understand the sources of protection and ultimately develop more effective formulations, we review ideas about the mechanisms of protection, survey the literature searching for patterns of protection, and then compare the ideas to the data.

Graphical Abstract

[*]**Corresponding Author** Telephone: 919-962-4495. gary_pielak@unc.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acs.bio-chem.9b00675. Abilities of additives to protect proteins against vacuum drying (Table S1), abilities of additives to protect proteins against freeze–thaw stress (Table S2), abilities of additives to protect against freeze-drying (Table S3), and comparison of additives that protect LDH (Table S4) (PDF)

The authors declare no competing financial interest.

Biologics, such as protein-based drugs, are some of the most effective therapeutic treatments on the market.[1] However, these medications are inherently unstable and can easily degrade with time or changes to their environment.[2] The World Health Organization even varies its stability guidelines for biologic drugs to accommodate the temperature and humidity ranges of different regions.[2] Furthermore, biologics usually require transport and storage in refrigerated conditions, also called the cold chain, which adds additional challenges and expense to maintaining drug integrity.[3] Enzymes are similarly poised to revolutionize the industry of chemical synthesis; however, optimizing these reactions requires the stabilization of enzymes in nonphysiological, and often non-aqueous, environments.[4]

We summarize the ability of a variety of additives to protect enzymes and other proteins against common stresses of biologic and industrial enzyme manufacture, transport, and storage, specifically, vacuum-drying (desiccation), freeze-thawing, and freeze-drying. Many of the additives are already used as, or have the potential to serve as, excipients, non-active ingredients formulated with biologics to stabilize and protect the active ingredient.[5] More specifically, we define excipients as compounds appearing as such in the Appendix of the book chapter by Gokam et al. and mostly focus on protection data published after the book by Franks and Auffret.[6]

STRESSES

Vacuum-Drying (desiccation) Stress.

Removing water from biologic drugs and industrial enzymes drastically reduces the weight of these products, which decreases shipping costs. Water can be removed from liquid formulations in a vacuum chamber, which is often combined with a centrifuge (e.g., a Speedvac).

Freeze-Thawing and Freeze-Drying Stress.

The chemistry of protein degradation usually requires the inherent motion in liquid formulations. Therefore, it can be advantageous to freeze enzymes, but freeze-thawing is often damaging.[7] In addition to vacuum-drying, water can be removed from frozen samples via lyophilization, resulting in both freeze-thawing and vacuum-drying stress.

MECHANISMS OF PROTECTION

Several mechanisms for protection from the stresses mentioned above have been considered. Some groups suggest that vitrification, when additives form an amorphous glass rather than ordered crystals as they dry, protects the enzymes by encasing them. This idea is often investigated using scanning calorimetry to observe the glass transition temperature.[8-15] Additional support for this idea comes from the vitrification observed in desiccation-tolerant organisms.[14,16,17] However, like all of the mechanisms, this idea is not universally accepted.[18,19]

Crowe et al. suggest that preferential hydration protects proteins against freezing-induced inactivation.[7] The idea behind this exclusion is that in aqueous solution, cosolutes such as simple sugars and compatible osmolytes are repelled by the protein backbone, favoring the more compact folded form of globular proteins, which stabilizes the protein by increasing its standard-state free energy of unfolding.[20,21] These repulsive cosolute–protein interactions have been especially well quantified for sugars.[21] Stability could also be increased via hard-core repulsions caused by high concentrations of inert cosolutes, which favor the compact native state of globular proteins.[22,23] It is unclear, however, if the effects of preferential hydration or macromolecular crowding extend to the dry state.

Some investigators suggest that inhibiting aggregation during vacuum drying,[24-28] freeze-thawing,[29] and freeze-drying is protective.[30] Chakrabortee et al. propose the molecular shield hypothesis, in which shield molecules use physical interference to reduce the frequency of cohesive interactions between potentially aggregating species.[26] This protection is conferred through nonspecific interactions. From efforts using solvatochromic dyes, Ferreira et al. suggest that the shield effect of protein additives called dehydrins arises from changes in water structure.[31] Several studies consider this hypothesis directly[24,26,29] or describe a similar phenomenon.[32,33]

Other groups report damage to enzyme structure from these stresses. Circular dichroism spectropolarimetry, Fourier transform infrared spectroscopy, and nuclear magnetic resonance-detected amide-proton exchange have been used to observe damage to secondary

structure.[28,33-37] Others report inhibition of enzyme-subunit dissociation[19,33,38,39] and suggest that maintenance of quaternary structure can compensate for a lack of water replacement during drying.[19,39] Several studies attribute dissociation to the precipitation of sodium phosphate buffer during freezing and the concomitant acidification of the sample.[39-42] However, this explanation fails to account for dissociation under other buffer conditions or other stresses.

Seguro et al. suggest that cryo-denaturation is caused by ice crystal formation.[41] In addition to freeze–thaw protection, a few groups report inhibition or slowing of ice crystal formation by additives that may, in turn, prevent enzyme damage.[11,33,43] Hillgren et al. postulate that surfactants may protect enzymes by covering ice crystals as they form,[11] which could explain why 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), polidocanol, polyethylene glycol (PEG) dodecyl ether, polysorbate, and sucrose fatty acid monoester protect some enzymes against freeze-thawing- and freeze-drying-induced inactivation.[11,19,30,44,45] Nevertheless, this explanation fails to explain protection from vacuum-drying damage.

ADDITIVES AND PROTECTION FROM VACUUM-DRYING STRESS

We compiled reports of enzyme protection during vacuum drying (Table S1). The highest degree of protection for each additive is further summarized in Figure 1. Focusing on the strongest protection tends to increase false positives. This approach, however, helps decrease the number of false negatives, which are likely because many studies test only a single concentration or mole ratio.

The stress of vacuum-drying resembles anhydrobiosis. Therefore, interrogating desiccation-tolerant organisms could result in the discovery of new excipients. Many such creatures, including *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Anopheles gambiae*, use the nonreducing disaccharide trehalose to combat desiccation stress.[27,46,47] As might be expected, heterologous expression of a trehalose transporter improves the desiccation tolerance of Chinese hamster ovary cells,[48] and trehalose biosynthetic genes improve the drought resistance of rice.[49] *In vitro* studies show that high concentrations of trehalose protect lactate dehydrogenase (LDH) against vacuum-drying-induced inactivation.[9,14,24,50,51] Trehalose also protects the activity of the restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII.[8,52]

Most sugars tested have some ability to protect enzymes against desiccation. The sucrose polymer Ficoll 70 fully protects LDH, although a higher grams per liter concentration is required than for trehalose.[51] The glucose polymer dextran 20, the disaccharide sucrose, and the sugar alcohol mannitol partially protect LDH in a concentration-dependent manner.[51] Additionally, Ficoll and dextran reduce the level of desiccation-induced aggregation of the water soluble T-REx293 proteome.[26] Maltodextrin and raffinose protect the restriction enzyme *Eco*RI against desiccation-induced inactivation, although protection was not quantified.[8] The disaccharide maltose partially protects LDH activity at intermediate concentrations, but protection decreases at both low and high concentrations.[51] Of the sugars tested, only glucose has no protective ability.[51] The protective ability of sugars may depend

on the absence of reactive carbonyl groups.[51] Finally, it is important to bear in mind that there are closely related studies that we have not included because the identical drying method was not used. For instance, Colaco et al.[53] and Carpenter and Crowe[54] studied air-dried, rather than freeze-dried, samples. Although we do not discuss these results or include them in Figure 1, they are generally consistent with the statements above and are included in Table S1.

The sugars trehalose, raffinose, mannitol, and glucose are also compatible osmolytes, a class of molecules that restore homeostasis in organisms under osmotic stress.[55,56] However, only sugar-based compatible osmolytes protect enzymes against desiccation. The compatible osmolyte and sugar alcohol mannitol has some ability to protect LDH, but even at high concentrations, it protects only a fraction of the enzyme.[51] The compatible osmolyte and amino acid glycine, along with its methylated form, betaine, do not protect LDH.[51]

Although the sugar polymers dextran and Ficoll are protective, the synthetic polymer polyethylene glycol has no ability to protect LDH against vacuum-drying regardless of concentration.[51] Polyethylenimine provides partial protection to LDH, and polyvinylpyrrolidone protects *Eco*RI during vacuum-drying, although protection of the latter was not quantified.[8,57] These mixed results suggest that hard-core excluded volume from macromolecular crowding[23] in solution does not confer desiccation tolerance.

Organisms across all kingdoms of life also synthesize intrinsically disordered proteins[58] in response to stress.[59] These proteins are often classified as hydrophilins, defined by Garay-Arroyo et al.[60] as having both a high glycine content (>6%) and a hydrophilicity index[61] of >1.0. Late embryogenesis abundant (LEA) proteins are the best known of this family. Their heterologous expression increases the desiccation tolerance of yeast and the hyperosmotic tolerance of human cells.[62,63] During vacuum-drying, LEA proteins reduce the level of desiccation-induced aggregation of the water soluble T-REx293 and *Aphelenchus avenae* proteomes and partially protect ADP-glucose-pyrophosphorylase and glucose-6-phosphate dehydrogenase activity in the soluble leaf proteome of *Arabidopsis thaliana*.[25,26,28] *In vitro*, LEA proteins protect LDH, fumarase, and citrate synthase activity and inhibit the latter's aggregation.[24,26,28,50,62,64,65] Additionally, formulation with LEA proteins reduces the loss of fluorescence of the red fluorescent protein mCherry upon vacuum-drying and rehydration.[26]

Another family of intrinsically disordered proteins, cytosolic abundant heat soluble (CAHS) proteins, is required by tardigrades to survive desiccation.[14] Heterologous expression of these proteins increases the desiccation tolerance of both *Escherichia coli* and *Saccharomyces cerevisiae*, and *in vitro*, CAHS proteins protect LDH against desiccation.[14,51] Mitochondrially abundant heat soluble proteins from tardigrades increase the hyperosmotic tolerance of human cells, but these proteins have not yet been studied as protectants of purified proteins.[63]

Despite having no link to desiccation tolerance, many other proteins protect enzymes against vacuum-drying *in vitro*. The most common globular protein control for vacuum-drying experiments, bovine serum albumin (BSA), protects the activity of LDH and citrate synthase

and also prevents aggregation of the latter, although it does not protect the activity of fumarase or the fluorescence of mCherry [14,24,26,50,51,62,64]. The disordered bacterial signaling protein flgM protects LDH against vacuum-drying-induced inactivation more effectively than CAHS proteins.[51] Ubiquitin and an SH3 domain are similarly effective.[51] α-Crystallin and β-lactoglobulin have some protective ability.[28,50] Ribonuclease A, thaumatin from pathogenesis-related protein family 5,[66] and the chaperone HSP70 have no protective ability,[26,28,62] but this result could arise from an insufficient amount of additive.

The fact that so many proteins with well-studied functions and no known link to desiccation tolerance protect enzymes against vacuum drying-induced inactivation suggests that protection is not conferred by a particular amino acid sequence.[51] Rather, protection may be a general property of proteins.[51] Along these lines, it has been suggested that the ability to survive repeated cycles of hydration and dehydration is a prerequisite for the emergence of life.[67]

ADDITIVES AND PROTECTION FROM FREEZE–THAW STRESS

We summarized the data using the fewest freeze–thaw cycles reported in each paper (Table S2). The highest degree of protection reported for each additive is summarized in Figure 2. However, it is important to use caution when comparing these studies because many parameters, including cooling rates,[11,12,44] the enzyme batch, and the sample volume, can affect the results.

In cells, trehalose protects both membranes and proteins against freeze–thaw damage.[68] Exogenous trehalose increases the tolerance of a variety of cells and organelles, including *Lactobacillus bulgaricus*, *S. cerevisiae*, isolated thylakoids, and ram spermatozoa.[69-72] Heterologous expression of the *Staphylococcus aureus* hemolysin transmembrane pore allows trehalose uptake and improved cryopreservation of 3T3 fibroblasts and human keratinocytes.[73] Reports of *in vitro* enzyme protection, however, are inconsistent. There are conflicting reports concerning the cryoprotection of LDH,[9,19,45,74-77] and trehalose fully protects phosphofructokinase[74] but fails to protect lipoprotein lipase.[51]

Inconsistent results are reported for many sugars. One study reports full protection of LDH by glucose, but another reports no cryoprotection.[19,45] The same studies test three cyclodextrin variants, β-cyclodextrin, 2,6-di-*O*-methyl-β-cyclodextrin, and hydroxypropyl-β-cyclodextrin, and report no protection to full protection of the enzyme.[19,45] Most studies report full protection of LDH by sucrose,[19,74-76,78] but other experiments report incomplete protection of LDH and phosphofructokinase.[76,79] Similarly, maltose fully protects LDH and partially protects phosphofructokinase against freeze–thaw stress.[74] Dextran fully protects both catalase and LDH,[19,80] and Anchordoquy et al. report full protection of LDH by Ficoll.[19] Despite this variability, there is at least one report of full protection for every sugar molecule tested. It is likely that reports of partial or no protection result from using an insufficient amount of sugar, inhospitable buffer conditions, or other easily altered conditions.

The sugars trehalose, glucose, and cyclodextrin are also compatible osmolytes, and like sugars, reports of compatible osmolyte protection are inconsistent. Two reports observe partial protection of LDH activity by betaine, one of which also reports protection of LDH structural integrity via tryptophan fluorescence.[62,75] However, other experiments report no protection of LDH activity but full protection of phosphofructokinase activity by betaine.[75,76] Similarly, ectoine and its derivatives demethylectoine, homoectoine, and hydroxyectoine provide full or partial protection to LDH and full protection to phosphofructokinase.[74-76] Glutamate partially protects alcohol dehydrogenase, malate dehydrogenase, and pyruvate kinase.[40,76] Other studies, however, report full, partial, and no protection of LDH,[40,41,45] and one study reports no protection of glucose-6-phosphate dehydrogenase.[40] Glycerol partially protects catalase activity and fully protects LDH activity.[75,80] The N-acetylated diamino acids N^{α}-acetyllsyine, N^{ε}-acetyllsyine, N^{α}-acetylornithine, and N^{δ}-acetylornithine fully protect the structure of LDH as assessed by tryptophan fluorescence but only partially protect its activity.[75,76] The opposite is true of proline, which fully protects LDH activity but only partially inhibits its structural perturbation.[35,75] Trimethylamine *N*-oxide (TMAO) fully protects LDH activity and structure, but there is only one report.[76] Like sugars, there is at least one report of full protection for every compatible osmolyte tested. Again, it is likely that reports of partial or no protection result from insufficient compatible osmolyte concentration, inhospitable buffer conditions, or other easily modified conditions.

Similar to their behavior in desiccation tolerance, intrinsically disordered LEA proteins fully protect LDH and citrate synthase in vitro and partially protect ADP-glucose-pyrophosphorylase and glucose-6-phosphate dehydrogenase in the soluble leaf proteome of *A. thaliana*.[24,26,28,29,43,62,77,79,81,82] Two hydrophilins outside the LEA protein family also protect LDH.[77,83] Futhermore, an intrinsically disordered CAHS protein protects lipoprotein lipase against freeze–thaw inactivation.[51] On a molar basis, all of these intrinsically disordered proteins are more effective protectants than the globular protein BSA. Nevertheless, BSA protects the enzymes malate dehydrogenase and pyruvate kinase in addition to LDH, citrate synthase, glucose-6-phosphate dehydrogenase, and lipoprotein lipase.[24,29,39,40,43,51,62,78,79,81,82]

Among globular proteins, partial and full LDH protection is reported for lysozyme and ovalbumin.[29,78,79,81] Partial cryoprotection of LDH is reported for α-crystallin, apo-transferrin, cytochrome *c*, myoglobin, and phosphorylase *b*.[77,81] Ribonuclease A has no cryoprotective effect on ADP-glucose-pyrophosphorylase and glucose-6-phosphate dehydrogenase in the soluble leaf proteome of *A. thaliana*,[28] yet full, partial, and no protection are reported for LDH.[28,77,79,81,82] Both full cryoprotection and no cryoprotection are reported for proteins from pathogenesis-related protein family 5.[62,78] Like ribonuclease A, β-lactoglobulin has no cryoprotective effect on ADP-glucose-pyrophosphorylase and glucose-6-phosphate dehydrogenase in the soluble leaf proteome of *A. thaliana*,[28] but multiple groups report partial protection of LDH activity.[28,79,81,82]

Although full protection is reported for only a subset of these globular proteins, at least one study reports partial cryoprotection by each protein. It is likely that reports of no protection

result from insufficient concentration, incompatible buffer conditions, or other facile variables.

In addition to proteins, several synthetic polymers confer cryoprotection. Polyethylene glycol fully protects both LDH and phosphofructokinase.[11,18,19,33,45] Full cryoprotection and partial cryoprotection of LDH are reported for the related polymer, polyethylene glycol dodecyl ether.[11,19] Polidocanol, a short polyethylene glycol attached to a hydrocarbon chain, also partially protects LDH.[45] Polyvinylpyrrolidone fully protects LDH and catalase,[39,80] and polyethylenimine partially protects LDH against freezing and thawing.[57] The emulsifier polysorbate partially protects LDH.[19] Poly-L-lysine has no ability to protect LDH, but only one concentration was tested.[77]

Some studies report an increased level of enzyme protection with an increasing molecular weight of the synthetic polymer, sugar polymer, or LEA protein.[19,33,57,84] Given the ability of numerous monosaccharides, disaccharides, and other compatible osmolytes to protect enzymes against freeze–thaw stress, additional studies are needed to determine the mechanism of protection.

ADDITIVES AND PROTECTION FROM FREEZE-DRYING STRESS

In addition to vacuum drying, water can be removed from frozen samples via lyophilization, resulting in both freeze-thawing and vacuum drying stress. We have compiled reports of enzyme protection during freeze-drying stress (Table S3). The highest degree of protection for each additive is summarized in Figure 3.

Trehalose exhibits mixed results. Formulating trehalose with *Lactobacillus reuteri* CICC6266 cells protects native LDH and ATPase,[85] and trehalose provides full protection to β-galactosidase and L-asparaginase.[30,32] Alkaline phosphatase lyophilized with trehalose even retains activity for up to 84 days.[86] However, trehalose confers only partial protection to lipase and mannitol dehydrogenase.[87,88] Trehalose was most frequently studied with LDH, but at best, only partial protection is reported.[18,30,45,74,75,87] Furthermore, trehalose did not protect the enzymes phosphofructokinase and lipoprotein lipase against lyophilization-induced inactivation.[18,51]

A variety of results are also reported for other sugars. Sucrose fully protects LDH activity in *L. reuteri* CICC6266 cells and L-asparaginase activity in lyophilized formulations and inhibits aggregation of monoclonal antibodies.[32,85,89] However, others report that sucrose partially protects LDH and phosphofructokinase and does not protect ATPase in *L. reuteri* CICC6266 cells.[13,37,74,75,85] Maltose fully protects L-asparaginase but only partial protects LDH and phosphofructokinase.[13,32,74] Glucose fully protects β-galactosidase and L-asparaginase but does not protect LDH or phosphofructokinase activity.[18,30,32,45] Similarly, lactose fully protects alkaline phosphatase and L-asparaginase, but no or only partial protection is reported for lactate dehydrogenase.[13,18,32,86] Two studies examine five cyclodextrin variants (α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, 2,6-di-*O*-methyl-β-cyclodextrin, and hydroxypropyl-β-cyclodextrin), which confer, at best, only partial protection to β-galactosidase and LDH.[30,45] Additional reports examine the sugar alcohols

mannitol and sorbitol. Mannitol fully protects L-asparaginase; mixed results are reported for LDH, and no protection is observed for alkaline phosphatase and phosphofructokinase.[18,32,37,86] Sorbitol partially protects LDH, but no protection is observed when formulated with lipase.[87] Like these sugar alcohols, glucosylglycerol fully protects and glucosylglycerate partially protects mannitol dehydrogenase.[88] If we consider the greatest degree of protection, all of these sugars confer at least partial protection.

The sugars trehalose, glucose, cyclodextrin, mannitol, sorbitol, glucosylglycerol, and glucosylglycerate are also classified as compatible osmolytes. Reports of protection are less frequent among non-sugar compatible osmolytes. Glutamate and proline fully protect β-galactosidase against freeze drying, although glutamate only partially protects LDH and proline confers no protection to phosphofructokinase.[30,45,75] Betaine partially protects phosphofructokinase but does not protect LDH or lipase.[74,75,87] However, derivatives of betaine, dimethylthetin and homodeanol betaine, have a limited ability to protect LDH and lipase.[87] Similarly, ectoine provides no protection to LDH but partially protects phosphofructokinase. Its derivative hydroxyectoine partially protects both enzymes.[74,75] Glycerol is the only compatible osmolyte with no documented protective ability, at least in limited studies with LDH and phosphofructokinase.[75]

Few proteins have been studied as protectants during freeze-drying and subsequent storage. Anchordoquy et al. report that BSA fully protects LDH.[39] Piszkiewicz et al. report that BSA partially protects, and a CAHS protein fully protects, lipoprotein lipase.[51] Furthermore, several enzymes protect themselves against freeze-drying with increasing concentrations.[30,39,45] Additional work is needed to determine if other proteins that protect enzymes against vacuum-drying and freeze-thawing stress are as protective against freeze-drying-induced inactivation.

Slightly more data are available for protection by synthetic polymers. Polyvinylpyrrolidone fully protects both catalase and LDH.[39,90] Polyethylene glycol confers partial protection of β-galactosidase, partial or no protection of LDH, and no protection of phosphofructokinase.[18,30,45] Polyethylenimine partially protects LDH activity.[57] Polidocanol is ineffective against freeze-drying.[30,45] Nevertheless, these studies are too limited to draw robust conclusions.

PROTECTION BY MIXTURES

Several groups report levels of protection from mixtures that are greater than those of the sum of the individual additives. Goyal et al. report protection of LDH and citrate synthase against desiccation-induced inactivation by a mixture of LEA proteins and trehalose.[24] Tamiya et al. report synergistic protection of LDH, malate dehydrogenase, glucose-6-phosphate dehydrogenase, and pyruvate kinase against freeze–thaw stress by BSA combined with sodium glutamate.[40] Mannitol and glycine formulated in 1:1 5% (w/w) ratios protect LDH from freeze-drying inactivation.[12] This study, however, does not investigate the protective ability of these compatible osmolytes individually and shows that the concentration of buffer in the original solution has a strong effect on protection. Mattern et al.[10] sought to assess the protective ability of vitrification using glasses made from amino acids, but most amino acids form crystals. These authors found that arginine forms a glass

with a low water content when mixed with phenylalanine. The low water content is important because the authors posit that residual water adversely affects protection. Phenylalanine and arginine together protect LDH against vacuum drying inactivation and inhibit granulocyte colony-stimulating factor aggregation, but these amino acids are not tested individually.[10] The authors made similar observations about mixtures of maltose or sucrose and phenylalanine.[91] Carpenter et al. observed that polyethylene glycol, which protects against freeze–thaw damage, can be combined with trehalose, glucose, or lactose, each of which protects against water removal, to protect LDH and phosphofructokinase against freeze-drying.[18] Miller et al. report that combining borate with trehalose synergistically improves the long-term storage of LDH after vacuum-drying.[9]

Each report investigates a limited set of potential synergistic interactions. More thorough studies are needed to confirm the conclusions and understand the mechanism(s) leading to synergism.

ANALYSIS AND CONCLUSIONS

Dozens of studies report on the ability of additives to protect enzymes against vacuum-drying, freeze–thaw, and freeze-drying stress, but there remains much we do not understand. For instance, although several mechanisms have been advanced, few studies have been designed to tell one mechanism from another. Even so, uncovering the mechanism(s) will be difficult because some molecules have no protective ability in one report and full protective ability in another. Some of these results may be false negatives because an insufficient quantity of additive is used. Given the absence of obvious patterns, it is likely that different test proteins are protected via different mechanisms. For instance, some test proteins are more sensitive to stress-induced changes in tertiary or quaternary structure while others are more prone to stress-induced aggregation. Additionally, most papers study only a small subset of additives. Another caveat is that additives with the largest number of reported protective effects may reflect the number of studies that used them rather than better protection.

Looking across Figures 1-3 shows that representatives from all classes of additives can have a protective effect. Given the wide range of chemical properties, this generalization provides support for the molecular shield mechanism. The most striking observation is that trehalose is generally protective against all three types of stress. Enhanced hydrogen bonding between trehalose and test proteins is a partial, but not full, explanation.[92]

Another general observation comes from comparing the results from vacuum-drying (Figure 1) to the those from freeze-drying (Figure 3) and freeze-thaw (Figure 2) experiments. Except for trehalose, compatible osmolytes, which stabilize proteins in solution[56] and in cells,[93] protect poorly against vacuum-drying (Table S1), suggesting that simple ideas about protein stability in solution cannot necessarily be related to this type of protection and perhaps that inactivation arises from protein–protein interactions.

We performed a separate analysis on LDH, the enzyme with the most protection data. We focused on additives reported to protect >90% of the activity. Where multiple concentrations

were tested, we focused on the lowest concentration affording full protection. The data are summarized in Table S4. There are too few data to analyze freeze-dry inactivation.

Data on LDH protection were divided into effects of small (<1 kDa) and large additives (>1 kDa). The median ratios are reported in Table 1 with one exception. The exception is that of trehalose, because it is the only small molecule that fully protects against desiccation stress.

Analysis of the data in Table 1 shows that large molecules are generally more effective than small molecules at protecting against both desiccation and freeze–thaw inactivation, which suggests a macromolecular effect. For freeze–thaw stress, however, protection cannot be attributed to hard-core repulsions because the concentrations of the protein additives studied [always ≤1 g/L (Table S4)] reflect minute fractions of the total volume. Freeze-thaw protection by large molecules may arise from nonspecific chemical interactions. Such interactions would appear to be concentration-dependent because LDH, malate dehydrogenase, alcohol dehydrogenase, pyruvate kinase, phosphofructokinase, and β-galactosidase all retain more activity when frozen at higher concentrations in the absence of additives.[30,39,40,42,45,74]

Upon comparison of desiccation data and freeze–thaw data, it is important to bear in mind that the concentration of the additive increases massively during desiccation but changes little during the freeze–thaw process. Trehalose, BSA, and Ficoll protect against both desiccation and freeze–thaw stress. However, more macromolecular additive is required to protect against desiccation-induced inactivation (Table1), suggesting that desiccation is more damaging. This idea is bolstered by examining the additives that protect against freeze-dry stress but not desiccation stress: the reducing sugars, dextran, maltose, and glucose. This lack of protection may arise from covalent modification by these sugars (glycation, a known problem in formulation)[94] as their concentration increases during desiccation. Further support for the suggestion that different mechanisms operate for freeze-thaw- and desiccation-induced inactivation comes from the observation that lysozyme denatures LDH in concentrated solutions, but dilute lysozyme solutions can fully protect against freeze–thaw inactivation.[51]

Nevertheless, the simple idea that polymers protect better against desiccation-induced inactivation is not valid because neither dextran nor PEG protects against desiccation. For Ficoll, the difference could arise from the reactivity of reducing linkages as suggested above, but it is unclear why PEG offers no protection against desiccation-induced inactivation but full protection against freeze–thaw stress.[51]

As stated at the beginning of this Perspective, cosolute-induced preferential hydration and/or hard-core crowding effects along with the molecular shield hypothesis involving hydrogen bonding between the protectants and the test proteins are the most popular proposed mechanisms. With respect to the ideas behind hydrogen bonding and the molecular shield hypothesis, it must be remembered that attractive interactions between cosolutes and the backbone of globular proteins are destabilizing because unfolding exposes more backbone. These attractive interactions are what make urea a protein denaturant.[21] In terms of ideas about hydration and crowding, we need to know if these concepts are operational in the solid

state. Techniques that can interrogate protein structure and stability in both solutions and solids include Fourier transform infrared spectroscopy,[16] solid-state NMR spectroscopy,[95] and synchrotron circular dichroism spectropolarimetry.[96] Although challenging, more such studies will be required to understand protection.

Most importantly, a direct comparison of a larger number of these additives under the same conditions using a variety of test proteins is needed. These suggestions should also be applied to the investigation of synergistic interactions to enhance protection. Systematic, hypothesis-driven studies utilizing many additives and several test proteins will determine which, if any, of the mechanisms are operative and ultimately lead to more effective formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Elizabeth Pielak for comments on the manuscript and Jhoan Aguilar for designing and preparing the Table of Contents graphic.

Funding

Our research is supported by the National Science Foundation (Grants MCB 1410854 and CHE 1607359 to G.J.P.) and the National Institutes of Health (R01GM127291). G.J.P. thanks the K. C. Wong Education Foundation for travel support. S.P. thanks the UNC Graduate School for a dissertation completion fellowship.

ABBREVIATIONS

BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CAHS	cytosolic abundant heat soluble
LDH	lactate dehydrogenase
LEA	late embryogenesis abundant
mCherry	red fluorescent protein
PEG	polyethylene glycol
SDS	sodium dodecyl sulfate
TMAO	trimethylamine *N*-oxide

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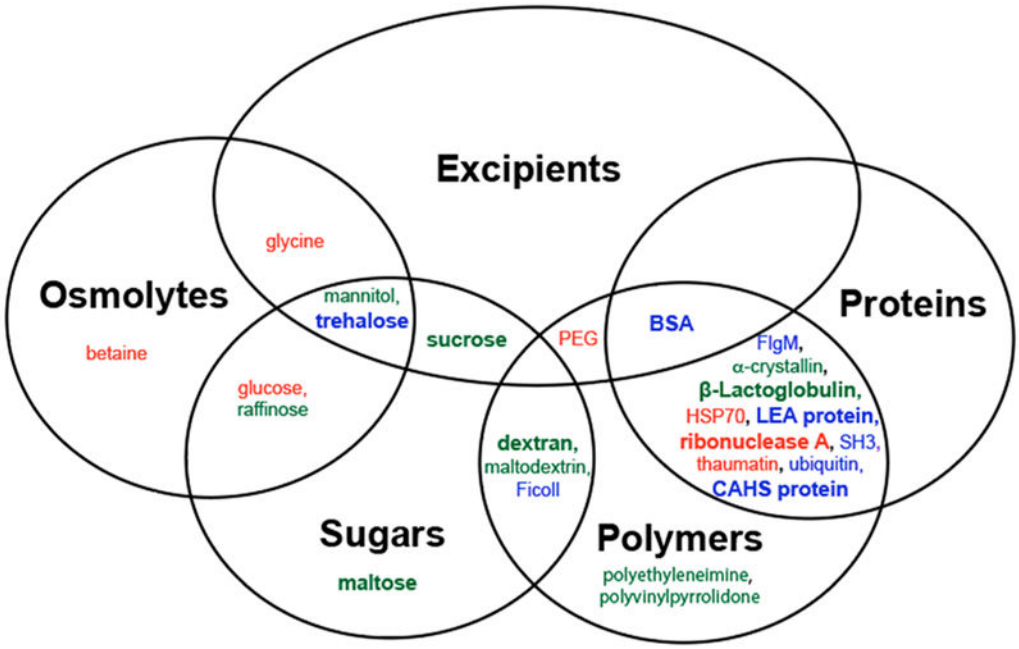

Figure 1.

Venn diagram for protection from vacuum-drying stress. Additives in blue have at least one report of full protection. Additives in green have no reports of full protection but at least one report of partial protection. Additives in red have no reports of protection. A bold font represents two or more reports.

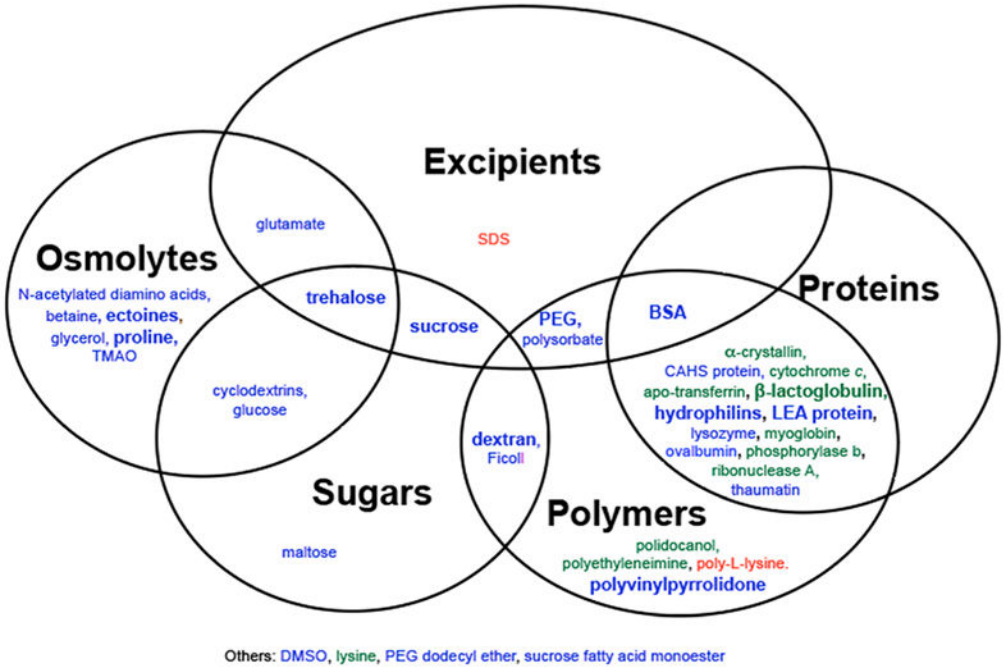

Figure 2.

Venn diagram for protection from freeze–thaw stress. Additives in blue have at least one report of full protection. Additives in green have no reports of full protection but at least one report of partial protection. Additives in red have no reports of protection. A bold font represents two or more reports. DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

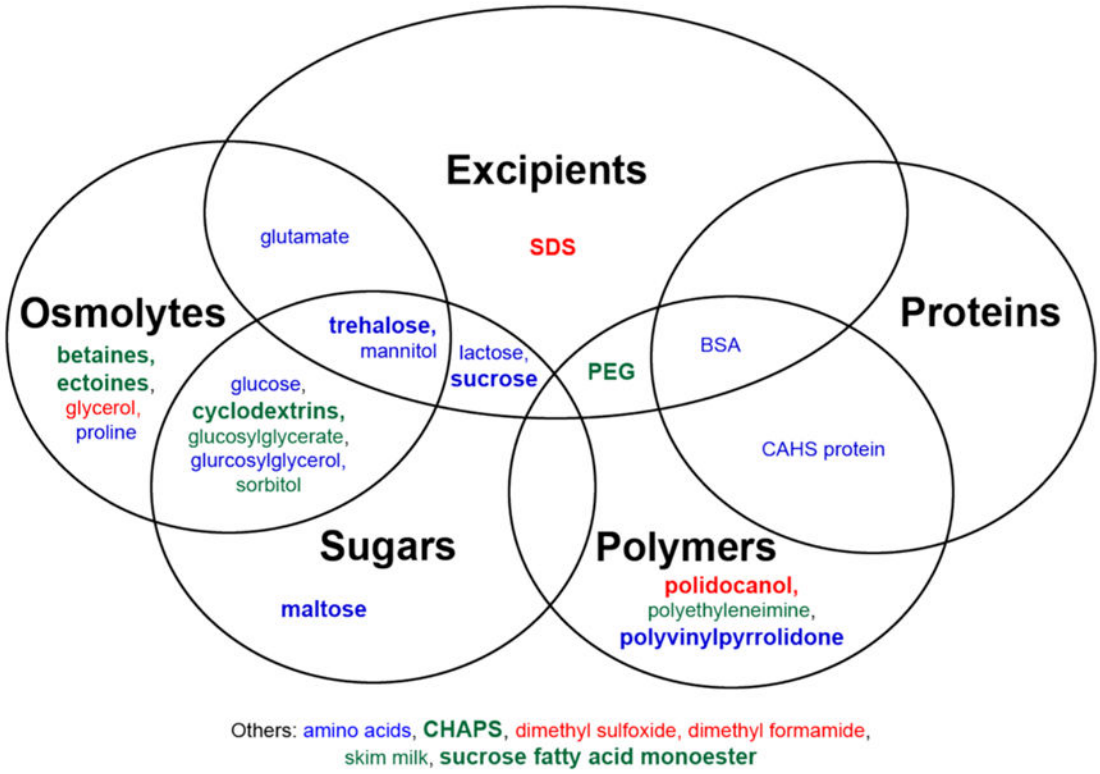

Figure 3.

Venn diagram for protection from freeze-drying stress. Additives in blue have at least one report of full protection. Additives in green have no reports of full protection but at least one report of partial protection. Additives in red have no reports of protection. A bold font represents two or more reports. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate.

Table 1.

Analysis of Additives That Protect LDH[a]

		additive:LDH	
stress	**additive**	**mole:mole**	**gram:gram**
desiccation	large	4×10^2	80
	small (trehalose)	1×10^5	250
freeze-thaw	large	25	4
	small	2×10^6	3×10^3

[a]See the text for details.