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Extending the Shelf-Life of Lyophilized Protein Formulations: Amino Acids as Stabilizers and Early Detection of Amorphous Phase Separation

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Extending the Shelf-Life of Lyophilized Protein Formulations: Amino Acids as Stabilizers and Early Detection of Amorphous Phase Separation

Kelly Michelle Forney-Stevens

University of Connecticut, 2013

Lyophilized protein pharmaceuticals are not infinitely stable and have a limited shelf-life often necessitating refrigeration. Excipients are added to protect the product during both processing and storage; however, the mechanisms of stabilization are still not fully understood. The goal of this research was to better understand critical factors in stabilization of dried proteins, such that long-term, ambient-temperature storage can be achieved. There were two main objectives:

1) Investigate amino acids as excipients to potentially extend the shelf-life of lyophilized proteins.

2) Refine a method for detection of amorphous-amorphous phase separation to better assess potential long-term stabilization issues.

First, the stability of sucrose-based protein formulations was evaluated in the presence of amino acids. Stability of two proteins were improved by the addition of all amino acids studied, over a wide concentration range. Molar volume and polarity of the amino acids influenced the stabilization of the proteins. There was no correlation between measures of global mobility and stability; however, in some cases decreased local mobility was accompanied by improved stability. Stabilization was not well correlated with a decrease

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in the system free volume. While no clear predictive measures were identified, amino acids in general showed a notable ability to improve storage stability of dried proteins.

Second, a confocal Raman microscopic technique was optimized to more efficiently detect amorphous-amorphous phase separation in lyophilized protein formulations. By assessing various instrument settings, a previous method was refined to significantly reduce experimental time. Phase separation was detected in protein systems where phase separation had previously been suspected. Raman microscopy has the potential to be used as an early indicator of suboptimal stability by identifying phase separation without the need for long-term stability studies.

The establishment of amino acids as stabilizers for lyophilized proteins and the identification of an indicator for potentially poor stability in these formulations contribute to the long-term goal of achieving more stable proteins. Greater stability of therapeutic proteins will facilitate their distribution and use globally by military troops and by patients in less-developed countries, where product quality is often compromised by the lack of a cold-chain and proper storage conditions.

Extending the Shelf-Life of Lyophilized Protein Formulations: Amino Acids as Stabilizers and Early Detection of Amorphous Phase Separation

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B.S., University of Pittsburgh, 2006

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

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at the

University of Connecticut

2013

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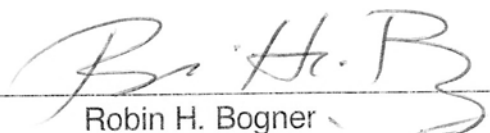
APPROVAL PAGE

Doctor of Philosophy Dissertation


Extending the Shelf-Life of Lyophilized Protein Formulations: Amino Acids as Stabilizers
and Early Detection of Amorphous Phase Separation


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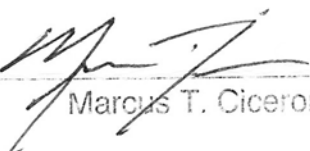
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Dedication

To my family and friends for all their continued support and encouragement, even when they had no idea what I was doing in Connecticut

&

To my husband, Ben, who was my rock throughout my graduate school career.

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Chapter 1

Objectives and Organization of Thesis

Introduction

Therapeutic proteins and vaccines are an important product class of drugs in human health care. However, due to both the chemical complexity and marginal stability of higher order structure, therapeutic macromolecules often present significant challenges in their development for pharmaceutical use. A pharmaceutical product must have adequate stability in ambient temperature storage during product shipping and distribution as well as long-term shelf-life storage stability for typically several years. Due to the inadequate stability of many proteins in aqueous solutions, a solid-state formulation is often necessary, where a solid is produced by drying techniques (e.g., freeze-drying). It is generally accepted that drying stabilizes the protein by decreasing molecular motion, thereby slowing all dynamic processes, including degradation. While both aqueous and solid-state protein formulations are susceptible to chemical and physical instabilities, the solid-state formulation usually has superior stability due to the minimization of degradation through limited mobility and the absence of water as a reactant(1, 2).

Formulation is often carried out by molecularly dispersing the protein in a protective matrix, providing mechanical immobilization and spatial separation of potential reactive sites. The entire system must remain in the same glassy phase in order to inhibit translational and relaxation processes. Excipients used to formulate the matrix usually have high glass transition temperatures, in order to effectively anti-plasticize global dynamics (increase the glass transition temperature, T_g) of the system. Below the glass

transition temperature of the matrix, the protein will be immobilized in the glass and the mobility of the reactive groups on the protein and in the environment will be greatly hindered, thus suppressing degradation reactions(3). Unfortunately, below the glass transition temperature motion does not stop, and both chemical and physical degradation of macromolecules can occur at unacceptable rates due to local or short-range rotational motions and group translational motion. In some cases local mobility may lead to global fluctuations(4). This suggests that storage of a macromolecule in a glassy matrix below the glass transition temperature does not necessarily guarantee an adequate shelf-life since mobility on a faster timescale than the glass transition may be effecting pharmaceutical stability(5).

Local motions within the glass, presumably resulting from intra-molecular reorientations occurring on a much shorter timescale (microsecond to nanoseconds), have been suggested to be controlling stability in systems well below T_g (6). Control of the dynamics has been supported by the observation that the addition of low levels of small molecule additives (i.e., glycerol and sorbitol) to disaccharide-based protein formulations were increased stability was correlated with a suppression of fast dynamics(7, 8).

As a result of the tendency for an amorphous solid to relax toward the more stable state, the components of the formulation may phase separate. If phase separation occurs between the protein and the stabilizing sugar and/or the low molecular weight compound, a protein-rich phase may be formed and the protein will no longer be

surrounded by a stabilizer-rich matrix(9). Without a suitable amount of stabilizer surrounding the protein, the molecular motions are no longer fully immobilized by the matrix and degradation will be more likely to occur during storage. Also, in order for the low molecular weight additive to fill in the empty spaces between the sugar and the protein, there must be phase compatibility between the low molecular weight compound and the sugar, meaning both excipients must be in the same amorphous phase as the protein(10). Therefore, determining if amorphous-amorphous phase separation is present in the formulation, after processing and during storage, will aid in the understanding of the stabilization mechanism as it relates to the chemical and structural properties of the low molecular weight compound.

A full understanding of the mechanism by which formulation components affect the fast dynamics of a system is needed in order to improve ambient temperature storage stability.

Objectives

There were two main objectives of this research:

1. To investigate the impact of low molecular weight compounds (i.e., amino acids) on the storage stability of model lyophilized sucrose-based formulations and to understand the relationship between the properties of the amino acids and various measures of mobility.

2. To optimize a confocal Raman microscopic technique to more efficiently detect amorphous phase separation in lyophilized protein formulations.

Organization of Thesis

In **Chapter 2**, a review of amino acids as excipients in solid-state protein formulations is presented. A background of amino acids used in aqueous protein formulations is given, with the goal of interpreting stabilizing mechanisms potentially relevant to solid-state formulations. This is followed by a discussion of amino acids that have been shown to improve protein stability during processing and storage. The potential for the future use of amino acids as stabilizing excipients in lyophilized protein formulations is discussed.

In **Chapter 3** the results of amino acids as stabilizing excipients in two sucrose-based protein formulations are presented. The purpose of this study was to explore the stabilizing effect of several different amino acids at one concentration on the long-term storage stability of lyophilized sucrose-based protein formulations. The amino acid properties (e.g., size, charge, polarity, hydrophilicity) were investigated to determine potential correlations with improvements in stability.

In **Chapter 4**, the results of the long-term storage stability of a sucrose-based protein formulation lyophilized with small molecule additives (i.e., amino acids, sorbitol) over a large concentration range is presented. The objective of this study was to investigate the extent of stabilization for amino acids and sorbitol at several concentrations and to

determine what concentration range of amino acid provided the highest stabilization for each additive. Potential correlations between the maximum stabilizing concentration for each additive and the additives size, charge and hydrophilicity were investigated.

In **Chapter 5**, measures of mobility and free volume in sucrose-based protein formulations lyophilized with amino acids was investigated. These results were evaluated to better understand the impact of “small molecule” additives on long-term storage stability and to assess potential mechanism(s) of stabilization by the amino acids.

The objective of **Chapter 6** was to further refine a Raman microscopic line mapping analysis for more routine use in the detection of amorphous-amorphous phase separation in freeze-dried protein formulations. The effects of sample preparation and various instrument settings on the efficiency and sensitivity of the analysis were evaluated. The optimal method was used to evaluate two protein systems where phase separation was previously suspected but never proven due to method limitation.

The overall results and significance of the dissertation are summarized in **Chapter 7**.

References

1. Maa Y-F, Prestrelski SJ. Biopharmaceutical Powders: Particle Formation and Formulation Considerations. *Curr Pharm Biotechnol*. 2000;1:283-302.
2. Rey L, May JC, editors. *Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products*. New York: Marcel Dekker, Inc.; 1999.
3. Yoshioka S, Aso Y. Correlations Between Molecular Mobility and Chemical Stability During Storage of Amorphous Pharmaceuticals. *J Pharm Sci*. 2006;96(5):960-81.
4. Hill JJ, Shalaev EY, Zografi C. Thermodynamic and Dynamic Factors Involved in the Stability of Native Protein Structure in Amorphous Solids in Relation to Levels of Hydration. *J Pharm Sci*. 2005;94(8):1636-67.
5. Luthra S. *Impact of Optimum Annealing on Chemical Stabilization of Model Amorphous Pharmaceuticals*. Storrs: University of Connecticut; 2007.
6. Cicerone MT, Tellington A, Trost L, Sokolov A. Substantially Improved Stability of Biological Agents in Dried Form: The Role of Glassy Dynamics in Preservation of Biopharmaceuticals. *BioProcess International*. 2003;V1(1):36-47.
7. Chieng N, Cicerone MT, Zhong Q, Liu M, Pikal MJ. Characterization of Dynamics in Complex Lyophilized Formulations: II. Analysis of Density Variations in Terms of Glass Dynamics and Comparisons with Global Mobility, Fast Dynamics, and Positron Annihilation Lifetime Spectroscopy (PALS). 2013.

8. Cicerone MT, Soles CL, Chowdhuri Z, Pikal MJ, Chang L. Fast Dynamics as a Diagnostic for Excipients in Preservation of Dried Proteins. *American Pharmaceutical Review*.22-7.
9. Padilla AM. Phase Separation in Freeze-Dried Amorphous Solids: Detection and Controlling Factors. Storrs: University of Connecticut; 2009.
10. Cicerone MT, Tellington A, Trose L, Sokolov A. Substantially improved stability of biological agents in dried form: The role of glassy dynamics in preservation of biopharmaceuticals. *Bioprocess International*. 2003;1:36-47.

Chapter 2

Amino Acids as Excipients in Solid-State Protein Formulations: Review and Discussion of the Potential to Improve Stability During Processing and Storage

Abstract

Amino acids are generally used to improve both solubility and stability in solutions of therapeutic macromolecules. Their use in solid-state protein formulations is often limited to buffering. However, recent research shows the potential for amino acids and other small molecule additives to enhance the long-term storage stability of dried pharmaceutical proteins. First, an overview of amino acids properties in the solution and solid state is presented. This review gives an overview of the use of amino acids in the solution-state, followed by a summary of current literature studies describing the use of amino acids in processing and on storage stability of dried protein formulations. Additionally, the mechanism of stabilization by the amino acids in the solid-state is explored.

1. Introduction

Macromolecules are highly susceptible to both chemical and physical degradation. As a result, pharmaceutical proteins are often dried (e.g., freeze-dried, spray dried, vacuum-dried) to extend their shelf-life, where the solid-state formulation often has superior stability relative to a solution(1). However, degradation processes still occur in the solid-state and stabilizing excipients are added to dried protein formulations to protect the protein during processing, shipping and long-term storage. The optimum type and amount of stabilizing excipient is generally considered to be dependent on the nature of the protein and its concentration in the final product. A trial-and-error approach to formulation is often used since the mechanisms of excipient stabilization in the solid-state are not fully understood.

Achieving an acceptable pharmaceutical shelf-life is highly dependent on excipient choice to sufficiently inhibit degradation reactions including deamidation, oxidation, dimerization and aggregation(2). Excipients such as disaccharides and polymers have been shown to provide adequate stabilization during both processing and storage at refrigerated temperature(3). Currently, there are no protein therapeutics that are stored at room temperature. Consequently, a protein formulation is considered to be pharmaceutically stable if it meets stability specifications for 15-18 months at refrigerated temperature (2-8°C)(4). Further improvements in stabilization approaches are necessary to achieve stabilization at room temperature over the usual two-year period. Moreover, distribution of therapeutic proteins to patients in

tropical climates without maintaining a cold-chain will require further stabilization efforts.

Solid-state stabilization of proteins has been the subject of previous reviews(5-10). Reviews of protein stabilization by amino acids focus on liquid formulations and only briefly address the solid-state(5, 6, 8, 9, 11-14). The purpose of this review is to focus on amino acids as stabilizing additives in solid-state protein formulations. A comparison of amino acid properties is presented, focusing on those specific properties considered to be important to the stabilization of therapeutic proteins. Stabilization of proteins in the solution state is discussed to identify potential mechanisms of stabilization that possibly apply to proteins in the solid-state. Ultimately, the effect of amino acids on the in-process stability and long-term storage stability of proteins in the solid-state is analyzed. The variety of proteins and their concentrations as well as additional excipients used in the various studies, make direct comparison of amino acid stabilization difficult. Still, common trends and findings emerge from the assembled literature. The ultimate goal of this review is to provide further insight into the use of amino acids to enhance the stability of solid-state protein therapeutics.

2. Amino Acids Properties

2.1 Solution-State Properties

From a formulation stand point; amino acids have a wide range of properties, which provide a large range of selection as potential stabilizers in pharmaceutical development. In order to understand how one amino acid may be more favorable over another amino acid as a stabilizing excipient, the chemical and physical properties of the most commonly investigated amino acids are compared (Table 1). Included in the comparison are ornithine and citrulline, which are often not considered part of the 20 essential amino acids but are often found in literature based on the structural similarities to arginine(15).

An amino acid is defined as a molecule with both a carboxyl group and an amino group bonded to the same (alpha) carbon that has a specific side chain (i.e., residue or R-group). The chemical and physical properties of amino acids are defined by the side chain characteristics. In solutions, amino acids are often classified according to the side chain composition, as follows: 1) charged, 2) hydrophobic and 3) polar(16). Other classifications may further group the amino acids based on the presence of sulfur or aromaticity(17).

Amino acids are zwitterionic due to the large difference in pKa of the carboxyl (pKa 1.8 – 2.5) and amino (pKa 8.7 – 10.7) groups. The zwitterionic nature makes them useful buffers in the pH range relevant to most protein formulations. The isoelectric

point (pI) of amino acids with nonionizable side chains ranges from 5.7 – 6.3, Table 1.(15, 18, 19). The basic amino acids have pI's around 10 and the acidic amino acids have pI's close to 3. While histidine is ionizable, it also has a pI of 7.6, which is the reason it is the most commonly amino acid buffer, particularly for protein formulations with a pI close to 7.6(20, 21).

The molecular weights of the most commonly used amino acids (i.e., the essential amino acids) vary over more than 100 Da (Table 1). The smallest essential amino acid is glycine, with a molecular weight of 75 g/mol and the largest is tryptophan with a molecular weight of 181 g/mol. The molecular weights of amino acids are comparable in size to excipients such as polyols (e.g., sorbitol, glycerol) and salts (e.g., KCl, CaCl₂) and smaller in size compared to polymers and disaccharides. The low molecular weights lead to potential issues in solid-state formulations (i.e., lower glass transition temperature, crystallization during processing) that will be discussed in a later section.

The hydrophilicity of the amino acids has been studied by many different methods and various scales are referenced in literature, each scale having its own mean and range of values. The hydrophilicity data shown in Table 1 were normalized from several of the most commonly referenced scales (e.g., Kyte and Doolittle) in order for direct comparison(22) over a range of 0-1, with 0 representing the most hydrophilic amino acid. These hydrophilicity values are also referenced in the National Center for Biotechnology Information (NCBI) amino acid database(19). The most hydrophobic

amino acid is isoleucine (1.0 on the normalized hydrophobicity scale) and the most hydrophilic amino acid is arginine (0.0 on the normalized hydrophobicity scale). It is important to point out that the studies to determine the hydrophilic/hydrophobic nature of the amino acids are based on the residues and are not the entire amino acid structure. Therefore, while the trend qualitatively will not change when considering the entire range of amino acids, it is quantitatively difficult to compare the hydrophilicity values to other excipient types.

Perhaps a better way of comparing the hydrophilic or hydrophobic nature of certain amino acids to other stabilizing excipients is by the potential side chain hydrogen bonds (accepting/donning). Excipients able to form hydrogen bonds are often added to protein formulations in both the liquid and solid-state, due to the potential to form hydrogen bonds with the surface of the protein, in a manner similar to water. For example, sucrose is highly used to stabilize therapeutic proteins due to its preferential hydration of proteins in liquid formulations(11). For the amino acids in Table 1, arginine has the highest potential, possibly forming a total of 7 hydrogen bonds (next is citrulline, which is structurally similar to arginine and can potentially form 6 hydrogen bonds)(19).

Dipole moment of the amino acid side chain was derived through a derivation of the net atomic charges and the molecular electrostatic potential (Table 1)(23). Side chain dipole moments range in values is from 0 D (alanine) to 9.07 D (lysine). Higher dipole moments are associated with amino acids having charged and polar side chains,

thereby they are also comparable to the hydrophilicity of the amino acid side chains(24).

We have discussed only a portion of the chemical and physical properties relevant to amino acids. A recent review provides a more in-depth summarization of the 20 naturally occurring amino acids with additional physicochemical, biological and electronic properties of the amino acids in solution(24). The specific mechanisms of degradation of amino acids as part of a protein has been described elsewhere in detail(14, 16, 17, 25) and is not a focus of this review.

2.2 Solid-State Properties

Measurement of solid-state properties of amino acids has been primarily limited to those of the crystalline compound. Specifically densities and melting temperatures have been reported in the literature for crystalline amino acids. Densities of dried crystalline amino acids range from 1.167 g/cm³ for lysine to 1.636 g/cm³ for aspartic acid(26). The variation in densities is due to the molecular differences in the side chain chemical structure. Melting temperatures of amino acids range from 160°C for glutamic acid to 343°C for tyrosine(24).

The thermal properties of select amino acids have been further studied by using calorimetric techniques(27, 28) in an attempt to group amino acids based on their thermogram characteristics. It was found that the thermal stability of crystalline amino acids is dependent on the decarboxylation reaction and as a result the thermal

stability is also a function of the amino acid side chain. Thus, the thermal behavior of amino acids in the solid state is highly dependent on the structure and can be grouped in a manner similar to the classification of amino acids in the solution state (discussed above). The thermal stability of the hydrophobic amino acids is as follows: valine < isoleucine < alanine < proline < methionine < phenylalanine < tryptophan, where tryptophan is considered the most thermally stable amino acid. The polar amino acids have increasing thermal stability as follows: asparagine < cysteine < glutamine < serine < glycine < threonine < tyrosine. The charged amino acids are grouped according to positive and negative side chains. Lysine has better thermal stability compared to arginine and aspartic acid has better thermal stability than glutamic acid. Both basic amino acids have better thermal stability than the acidic amino acids and as a group, the basic amino acids have the best thermal stability out of all amino acid groups. It is hypothesized that the positive charges of the basic amino acids hinder the decarboxylation reaction, contributing to the improved thermal stabilization compared with the acidic amino acids, where the negative charges actually promote decarboxylation(28).

3. General Uses of Amino Acids in Liquid Protein Formulations

Amino acids are often added to liquid formulations to enhance solubility, function as osmolytes, buffer the system pH, act as antioxidants, stabilize the native protein structure, and prevent aggregation/denaturation(14, 29). Histidine is the most widely used amino acid in liquid formulations, most commonly used as a buffer(20, 21). Commonly, histidine and methionine are used as antioxidants(11, 13, 14, 30, 31). For

example, methionine as an antioxidant was successful in reducing oxidation of an antibody formulation(30). Glycine, proline, and glutamate have frequently been added to function as osmolytes in solution(13, 32, 33).

Stabilization by amino acids in liquid formulations has been attributed to several types of mechanisms including preferential hydration and bonding directly with the protein(13). Several studies have attempted to further investigate the mechanisms of stabilization (discussed below). However, in most, if not all of the studies on “stabilization” of proteins in solution-state, the protein was subject to severely destabilizing conditions. This reduced experimental time, however, in many cases the stability was only suability studied. The conditions used were not necessarily relevant to pharmaceutical stability, which investigates stability over longer time periods and under less “destabilizing” conditions. However, the solution-state studies could potentially provided insight into stabilization mechanisms of the amino acids. A summary of the studies is presented in Table 2 and discussed in detail below.

3.1 Evaluation of the protein melting temperature (T_m)

The melting temperature (T_m) at which a protein undergoes a structural transition is often used to assess protein stability. Solution conditions that increase T_m are thought to stabilize the protein structure. One study showed that an increase of 1°C for a protein T_m has been associated with increased thermal stability(34). However, the same study noted that thermostability can also be achieved without changing T_m . Other thermodynamic properties need to be considered when comparing stability and

the melting temperature of a protein. For liquid therapeutic proteins, there is no clear relationship between T_m and pharmaceutical stability. Often the measurement technique and experiment conditions will affect the value of T_m (11). Therefore, T_m is of more interest for understanding the unfolding and denaturation properties of a protein in the presence of excipients.

Several literature studies have investigated the effect of the protein stability by evaluating T_m in the presence and absence of selected amino acids, with contradicting results(35-37). The T_m of a 5 mg/mL IgG1 solution containing 0.0625 M of various amino acids was evaluated using differential scanning calorimetry (DSC)(36). The authors concluded that the side chain charge played an important role in protein stabilization. Amino acids with basic side chains provided the greatest stabilization (i.e., largest increase in T_m), neutral amino acids provided some improvement in stability, and amino acids with acidic side chains provided no improvement in stability.

The effect of amino acids (0.2 – 0.7 M) on the T_m of cytochrome-c in destabilizing conditions (0.05 M citrate buffer, pH 3) was investigated by measuring the protein denaturation by UV-Vis spectroscopy(35). It was found that isoleucine, phenylalanine and leucine had no effect on T_m , while valine, serine, proline, methionine and glycine increased the T_m of the protein, thereby increasing the protein stability. For the same study it was found that arginine, lysine and histidine decreased the T_m , resulting in destabilization of the protein. In this study, hydrophobic amino acids “stabilized” the

protein better than the charged amino acids and in fact, arginine and lysine “destabilized” the protein.

The stability of an antibody solution (0.1M IgG1) in the absence and presence of a variety of amino acids (0.3 M) where shifts in T_m were monitored by intrinsic Trp fluorescence over a wide temperature range (10 - 85°C) and a wide pH range (3 - 8)(37). The addition of the seven amino acids studied either destabilized or had no effect on the protein stability compared with sucrose.

These inconsistent results point out that T_m is highly dependent on the method used in the determination. The calorimetric results concluded that the basic amino acids stabilized better than the neutral amino acids and acidic amino acids actually destabilized the protein(36). The UV-Vis results found basic amino acids to be destabilizing and the study measuring intrinsic Trp fluorescence found no stabilizing amino acids. It is apparent from these studies that the value of T_m does not necessarily predict the stability of the protein. However, the calorimetric study did not expose the protein to destabilizing conditions, compared with the UV-VIS and Trp fluorescence study that exposed the protein to extreme pH conditions.

From a pharmaceutical standpoint, the calorimetric study indicates the stabilizing potential of basic amino acids at higher temperatures. These authors attributed the positive charge of the side chain on the basic amino acids to the stabilization,

proposing the transient binding of the side chain to the protein makes unfolding less energetically favorable(36).

3.2 Evaluation of Stability at Elevated Isothermal Conditions

There have been a few studies regarding the stabilization of aqueous protein formulations in the presence of amino acids at elevated temperatures(38, 39). In one study, the activity of lysozyme (0.2 mg/mL) formulated in 50 mM sodium phosphate buffer and an amino acid (0.05 – 0.5 M) was evaluated after storage at 98°C for at least 30 minutes(38). This is well above the T_m of lysozyme, document to be between 66 - 74°C(11). After exposure of lysozyme-amino acid solutions to higher temperatures, the majority of the amino acids protected the protein from degradation at all concentrations. The higher amino acid concentrations stabilized lysozyme to the greatest extent, with arginine, ornithine, serine, and lysine providing the best stabilization. Lysozyme was destabilized at the lower concentration of alanine (0.05M), but stable at the higher concentration of alanine (0.2 M) and was not stable at any concentration of glycine. It was proposed that the amino acid side chain structure could be very important to the improvement in stability, where the positively charged amino acids (i.e., lysine, arginine) can prevent the aggregation of the protein more effectively than the neutral amino acids.

The stability (37°C /90 days) of IgG1 (10 mg/mL) formulated with several different amino acids (0.1 M) was evaluated for aggregation by size exclusion chromatography(40). Isoleucine, methionine, proline, phenylalanine, valine and

glycine significantly prevented aggregation after accelerated storage, while histidine and arginine had no effect on the stability. These findings do not support the studies that found the positively charged amino acids improved the thermal stability of the protein.

The chemical and physical stability of a recombinant protein (rHEGF, 0.1 mg/mL) at 50°C was studied in the presence of 0.2 M alanine, glycine, histidine or leucine and was compared with the degradation stability of the protein in the presence of only a disaccharide(39). The stability of rHEGF with an amino acid was comparable to the stability with either sucrose or trehalose.

The lack of agreement in the results of the studies may be due to the difference in the amino acid concentration, the difference in the proteins studied, and the types of stability measured. The effect of amino acids on thermal stability may also depend on the other formulation components, the specific interactions between the amino acid used, and the interactions with the macromolecule under investigation.

3.2 Stabilization Against Aggregation During Refolding

One literature study investigated the chemical stability of lysozyme (0.2 mg/mL) in the presence of various amino acids, ranging in concentrations (0.05 – 0.5 M). The fraction of refolded protein (Lysozyme) in the presence of 0.8 M urea was determined by UV-VIS spectroscopy and compared with previous thermal stability results(38). As with the thermal stability study, arginine, lysine, and ornithine stabilized lysozyme

against aggregation to the highest extent. In contrast, glycine, glutamine, aspartic acid, and glutamic acid all destabilized the protein in the presence of urea. Alanine had some stabilization at the lower amino acid concentration (0.05M), while threonine destabilized lysozyme at 0.05 M but provided a small amount of stabilization at 0.2 M. Valine, leucine, isoleucine, proline, and methionine all provided the greater stabilization at the higher concentration studied (0.2 M). The results of this study show that most of the amino acids protect lysozyme against thermal degradation much better than chemical degradation.

3.4 Conformational Stability

A study investigated the conformational stability of BSA (0.15 mg/mL) and lysozyme (0.15 mg/mL) in the presence of alanine and glycine(41). The amino acids were investigated over a range of 0.7 – 1.4M. It was found that the addition of the amino acid induced preferential hydration of the protein in solution (compared to a control), thereby providing stabilization of the structure.

3.5 Stabilization Mechanism of Arginine

The stabilization of various proteins by arginine has been attributed to several mechanisms, including preferential hydration, ionic binding, surface tension effects, and side chain interactions(13, 41-43). It was proposed (and tested by Trp fluorescence) that the conformation stability of an antibody (0.1 mg/mL IgG1) was stabilized by arginine (0.25 M) molecules “bonding” with the solvent-exposed regions of the antibody, providing stabilization(44). Another study found that arginine (0.2M,

0.5M) inhibited aggregation of lysozyme (10 mg/mL) by disrupting intramolecular interactions between the protein surface and arginine(45). From the observations discussed above, it seems that the charge of the arginine side chain made a large difference in the stabilization of a protein in the liquid state.

3.6 Stabilization Mechanism of Proline

Proline has been shown to be highly stabilizing, with regard to decreasing aggregation and improving chemical stability in several protein formulations(40, 46-49). The stabilization of two proteins, lysozyme (1 mg/mL) and bovine carbonic anhydrase (0.1 – 1 mg/mL), by proline was attributed to the amino acid behaving as a protein folding chaperone in solution(48, 50). Another study investigating the aggregation and activation stability of lysozyme (1 mg/mL) formulated with proline, found that higher concentrations of proline (0.5 M) improved the thermal stability compared to solutions containing no excipient(48). This was attributed to proline providing a hydrophobic microenvironment for the dissolved protein, thereby protecting the protein from inactivation and preventing denaturation. A similar study measured the chemical stability of KGF (0.5 mg/mL) at 37°C, and found the protein stability was improved at all concentrations of proline (0.2 – 3M), compared to the formulations without any proline(47). Another case study investigating the aggregative stability of an antibody formulated with 0.25 M proline concluded that aggregation was inhibited by proline. The authors proposed that the proline binds to the exposed hydrophobic domains of the protein, thus inhibiting any interactions(46).

A separate study with proline attributed the inhibited aggregation to a decrease in the osmotic pressure of the protein solution(32).

4. Amino Acids as Stabilizing Excipients in Solid-State Formulations

4.1 Stabilizing Challenges in Solid-State Formulations

Common methods of preparing solid-state therapeutics are through drying processes, such as spray-drying, freeze-drying and vacuum drying(51). Excipients are added to solid-state protein formulations to protect against processing conditions and to extend the long-term shelf-life stability(52). Amino acids have been used as cryoprotectants (to protect the protein during freezing), lyoprotectants (to protect the protein during drying), and stabilizers that protect the dried protein product during storage. As with most solid-state formulations, the excipient must remain in the same amorphous phase as the protein, in order to fully protect the protein from degradation(52). The mechanisms of stabilization are different depending on the phase of the process and during storage, indicating amino acids can assume multiple stabilizing roles.

4.2 In-Process Stabilization

The use of amino acids as buffers in the solid-state has been studied with regard to the T_g' (maximally freeze concentrate) with the addition of the amino acid(53). Amino acids are natural buffers due to their chemical structure, however, the T_g' of each amino acid varies and this greatly influences processing conditions. For example, in

freeze-drying the shelf temperature of the lyophilization cycle is depended on the T_g' of the product, where a lower T_g' requires a lower shelf temperature, resulting in extended drying times as a result of the decreased sublimation rate of water from the product(8). In order for excipients to stabilize a protein, both the protein and the stabilizer must remain in the same amorphous phase. Thus, it is important to adjust processing conditions to avoid crystallization.

Histidine has been widely studied as a buffering agent, and has been contributed to improved stability of lyophilized proteins(20, 54, 55). Compared with other amino acids, histidine also has one of the higher T_g' values (-32°C) of the amino acids studied, making it a good candidate for processing development. In comparison, the T_g' values of alanine and lysine have been measured below -60°C , while arginine and glutamine have T_g' values closer to -40°C . Both temperatures at which make freeze-drying difficult due to the low shelf temperature required. Glycine and glutamic acid have contradicting T_g' values reported, ranging from -63 to -37°C for glycine and -32 to -17°C for glutamic acid(8, 9, 53). Most often when formulated with other excipients and a protein, the values of the T_g' and collapse temperature are higher, allowing for higher shelf temperature during lyophilization. A more systematic evaluation of T_g' values for amino acid formulations would allow for more choice in formulation design, as the amino can also buffer the protein, potentially eliminating unnecessary excipients (e.g., salts).

The processing of proteins from the liquid state to the amorphous state results in a lot of stress on the protein due to the freezing and/or drying step of the process. During the freezing step of the lyophilization process, the formation of ice results in increased concentration of the solute within the interstitial space between the ice crystals. This potentially results in protein adsorption to the ice-air interface, leading to degradation(51). Amino acids have been shown to protect proteins during freezing stresses. For example, histidine (4-60mM), lysine (27mM), and glycine (66-660M), protected several monoclonal antibodies during freeze-thaw studies, where the protein maintained acceptable levels of degradation after freezing compared to the pure freeze-dried protein(54, 56).

One study compared amino acids as either bulking agents and stabilizers in a recombinant protein formulation (rAHF 3130 IU/mL). The authors found that the addition of lysine, glycine, and arginine as stabilizers (2% w/w), along with a bulking agent (i.e., mannitol) lead to collapse of the cake during the freeze-drying process and loss in stability. However, when glycine and alanine were added as bulking agents (8% w/w), the result was an elegant cake and a stable product, where activity was maintained after storage at 5°C for 18 months(57). Another study investigated the processing stability of lyophilized bovine liver catalase (4 mg/mL) formulated with a series of amino acids over a range of concentrations (0.1 – 5% w/w) in order to determine the effectiveness of amino acids as stabilizers during the drying process of freeze-drying. The activity of the enzyme was maintained when lyophilized with alanine, arginine, glycine, histidine, lysine, proline, serine, and threonine. Only

cysteine and valine destabilized the enzyme(58). The authors did not use any bulking agent, and, it is not clear if the amino acids remained in the amorphous state throughout processing.

Another systematic study of amino acids was performed to evaluate the physical characteristics and drying behavior of amino acids (0.12M) formulated with two proteins, LDH (164 units/mL) and rhG-CSF (0.35 mg/mL), after vacuum-drying and freeze-drying(59, 60). Only lysine, arginine, citrulline, and histidine showed a glass transition temperature (T_g), while all other amino acids studied (leucine, methionine, proline, serine, valine, threonine, phenylalanine, cysteine, glycine, and isoleucine) crystallized during processing(59). Mixtures of protein, amino acids and salts were also investigated. It was determined that arginine, phenylalanine, and phosphate salt resulted in a pure amorphous solid after vacuum drying. Amorphous amino acid-protein formulations were obtained after vacuum drying by adding salts. However, this would not be ideal for lyophilization, where salts will significantly decrease the T_g of the product.

Conformational stability of a freeze-dried monoclonal antibody (10 mg/mL) was assessed by spectroscopic analysis. Histidine and arginine at concentrations of 10-200mM improved the conformational stability and maintained the product structure after freeze-drying. Aspartic acid had no effect on the conformational stability, while glycine resulted in alteration of the native structure(61). Glycine acted best as a bulking agents, rather than a stabilizing excipient(57, 58).

4.3 Long-Term Storage Stabilization

There are few studies regarding the T_g of the amino acids in amorphous protein formulations(53, 62). Most likely this is due to the tendency for the amino acids to crystallize. However, it has been shown that when formulated with other excipients, crystallization of amino acids is usually inhibited. For example, crystallization was completely hindered when two amino acids were used in the protein formulation at 0.12M concentrations. However, it was also shown that citrulline, arginine, lysine and histidine are amorphous after freeze-drying and vacuum-drying with a protein and no other excipient(59).

There are few long-term storage stability studies of formulations containing amino acids as stabilizing excipients. It has been shown that the most improvement in storage stability is for mixtures of an amino acid with other stabilizing excipients. This is particularly true for arginine, which has shown poor stabilization as a sole excipient(53) but was shown to be an excellent stabilizer when mixed with other excipients, including: organic acids, acidic salts and other amino acids(53, 59, 60, 63). In the presence of acidic salts, arginine stabilized rHG-CSF against aggregation and LDH against activity loss(59). When LDH and rHG-CSF were formulated with a combination of arginine, mannitol and phenylalanine, the stability was greatly improved compared to formulations without mannitol(60). Another study showed LDH retained enzymatic activity when freeze-dried with arginine (160mM, 200mM) and either citric acid or tartaric acid(53). Improved stability of a freeze-dried protein (Greengreen F) was found in the presence of a mixture of arginine, glutamic acid and

isoleucine (10-100 mM). The authors found each amino acid had a different range of stabilization. The final mixture was adjusted to include the most optimal amount of each amino acid and successfully stabilized the stability of recombinant factor VIII(63).

The long-term storage stability of an antibody and enzyme (LDH) was improved in the presence of histidine as a stabilizing additive(20, 53, 54, 64). For all formulations, higher amounts of histidine (35 mg/mL) provided the best stability. For example, the activity recovery was greatest for lyophilized formulations of histidine and LDH. However, when LDH was formulated with organic acids, it was found that the protein stability did not change(53). Histidine was found to be highly stable as both a buffer and a stabilizing excipient by, comparable to sucrose(20).

4.4 Comparison Between Liquid and Solid-State Stability

Several literature attempts have been made to compare solution properties with solid-state properties(65, 66). Here, an attempt is made to relate the solid-state stability with the liquid stability. However, there are many differences that are not well understood between the solution state and the amorphous state (i.e., pKa and pH). It is proposed that stabilizing excipients in amorphous formulations protect the protein through one of two mechanisms. Thermodynamically the stabilizer hydrogen bonds to specific sites on the protein, where water would normal bind. This “water substitution” hypothesis is based on the excipient providing conformational stabilization and increasing the free energy of unfolding. The other mechanism, the

kinetic hypothesis (also known as the “glass dynamics” hypothesis), states that the protein is molecularly dispersed in the excipient matrix, where the excipient provides mechanical immobilizing of the protein’s reactive sites thereby physically preventing degradation from occurring. Most likely, stabilization occurs via a combination of both mechanisms(67).

The addition of small molecules (i.e., sorbitol, glycerol) to disaccharide-based protein formulations has been reported to improve the solid-state stability of proteins, relative to the disaccharide alone(68, 69). The small molecule are thought to inhibit degradation of macromolecules in the solid-state through a reduction in the free volume of the amorphous matrix and a resulting suppression of dynamics on a nanosecond timescale(70, 71). This would be consistent with the “glass dynamics” theory, where the reactive sites of the protein would be further immobilized in the amorphous matrix(67). It is suspected that amino acids could act in a manner similar to sorbitol and glycerol in the solid-state.

Most amino acid side chains have hydrogen bonding potential, (Table 1), where they could bind to the protein in the same manor as water, consistent with the “water substitution” hypothesis(67). This stabilization hypothesis supports the studies showing the secondary structure of various proteins with an amino acid was maintained in the solid-state(14, 54). For example, histidine, which has three available hydrogen binding sites along its side chain, successfully maintained the conformational stability of a monoclonal antibody, similar to how sucrose is proposed

to stabilize(54). Each arginine molecule can form seven potential hydrogen bonds, which is the largest number of hydrogen bonds among the amino acids listed in Table 1. The number of potential hydrogen bonds could also explain why the charged amino acids more successfully stabilize proteins in the solution state. Potentially, the mechanism of stabilization may be related to the solution state mechanism, where side chain interactions of the amino acids bind with the protein surface(62). Stabilization by the positively charged amino acids in the solution state was found for several studies. For example, the results of the melting temperature of liquid IgG1 solutions was increased in the presence of basic amino acids, where the authors proposed stabilization of the protein by the positively charged side chain, where unfolding was made unfavorable(36). Lysozyme was also protected by the positively charged amino acids after exposure to high temperatures and destabilizing solutes(38).

Interactions between freeze-dried formulations of antibody and histidine and antibody and arginine were studied using calorimetry techniques. It was found that ion-dipole, electrostatic and hydrogen bonding occurred between the protein and select amino acids. No interactions were found between glycine and the protein, which could be due to the lack of any potential hydrogen bonds(62). (Note from the paper, it is not clear if the glycine was partially crystalline.) Another study by the same group using solid-state NMR also found that the side chain of arginine directly interacted with the surface of a monoclonal antibody, for the freeze-dried arginine/protein formulations. Histidine also had some structural protective effects similar to arginine, however,

aspartic acid and glycine were less protective. The maximum amount of structure was retained at the highest concentrations of histidine and arginine used in the study(61). The authors found that weak interactions between the protein and the charged amino acid may help preserve the native protein structure, which is consistent with the preferential binding mechanism found in the solution state. In another study, it was postulated that the amphiphilic nature of arginine protected the protein (interleukin-2) after storage by binding to like regions of the protein(72). The conclusions of the solid-state studies are consistent with those in the liquid state.

Most likely the mechanism of stabilization is a combination of the “glass dynamics” hypothesis and the “water substitution” hypothesis. The extent of stabilization depends on the specific protein as well as the amino acid concentration and the processing conditions.

5. Summary

The wide range of chemical and physical properties of amino acids make them potentially useful in therapeutics protein formulations, where amino acids offer a wide choice of excipients that can act as bulking agents, buffers and stabilizers. The amino acids have the ability to stabilize the protein in the solid state through both direct interactions with the protein (i.e., hydrogen bonding) or through providing a rigid amorphous matrix and suppressing dynamics. Much more research needs to be conducted to properly evaluate the stabilizing effect of amino acids on the long-term storage of therapeutic formulations.

6. References

1. Rey L, May JC, editors. Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products. New York: Marcel Dekker, Inc.; 1999.
2. Lai MC, Topp EM. Solid-State Chemical Stability of Proteins and Peptides. *Journal of Pharmaceutical Sciences*. 1999;88:489-500.
3. Carpenter JF, Manning MC, editors. Rational Design of Stable Protein Formulations: Theory and Practice. New York: Kluwer Academic/ Plenum Publishers; 2002.
4. Bontempo JA, editor. Development of Biopharmaceutical Parenteral Dosage Forms. New York: Marcel Dekker, Inc.; 1997.
5. Jorgensen L, Hostrup S, Moeller EH, Grohgan H. Recent Trends in Stabilising Peptides and Proteins in Pharmaceutical Formulation - Considerations in the Choice of Excipients. *Expert Opinion*. 2009;6(11):1219-30.
6. Ohtake S, Kita Y, Arakawa T. Interactions of Formulation Excipients with Proteins in Solution and in the Dried State. *Advanced Drug Delivery Reviews*. 2011;63:1053-73.
7. Akers MJ. Excipient - Drug Interactions in Parenteral Formulations. *J Pharm Sci*. 2002;91(11):2293-300.
8. Wang W. Lyophilization and Development of Solid Protein Pharmaceuticals. *Int J Pharm*. 2000;203:1-60.
9. Liu J. Physical Characterization of Pharmaceutical Formulations in Frozen and Freeze-Dried Solid States: Techniques and Applications in Freeze-Drying Development. *Pharm Dev and Tech*. 2006;11:3-28.
10. Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. Factors Affecting Short-Term and Long-Term Stabilities of Proteins. *Advanced Drug Delivery Reviews*. 2001;46:307-26.
11. Wang W. Instability, Stabilization, and Formulation of Liquid Protein Pharmaceuticals. *Int J Pharm*. 1999;185:129-88.
12. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of Protein Pharmaceuticals: An Update. *Pharm Reseach*. 2010;27(4):544-75.
13. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein-Excipient Interactions: Mechanisms and Biophysical Characterization Applied to

Protein Formulation Development. *Advanced Drug Delivery Reviews*. 2011;63:1118-59.

14. Arakawa T, Tsumoto K, Kita Y, Chang B, Ejima D. Biotechnology Applications of Amino Acids in Protein Purification and Formulations. *Amino Acids*. 2007;33:587-605.

15. Lehninger AL, Nelson DL, Cox MM. *Principles of Biochemistry*. 2nd ed. New York: Worth Publishers; 1993.

16. Branden C, Tooze J. *Introduction to Protein Structure*. 2nd ed. New York: Garland Science; 1999.

17. Creighton TE. *Proteins: Structures and Molecular Properties*. 2nd ed. New York: W. H. Freeman and Company; 1993.

18. Orgovan G, Noszal B. The Complete Microspeciation of Arginine and Citrulline. *Journal of Pharmaceutical and Biomedical Analysis*. 2011;54:965-71.

19. NCBI. *Amino Acid Explorer*: United States Government; 2009.

20. Al-Hussein A, Gieseler H. Investigation of Histidine Stabilizing Effects on LDH During Freeze-Drying. *J Pharm Sci*. 2012;102(3):813-26.

21. Katayama DS, Nayar R, Chou DK, Valente JJ, Cooper J, Henry CS, et al. Effect of Buffer Species on the Thermally Induced Aggregation of Interferon-tau. *J Pharm Sci*. 2006;95(6):1212-26.

22. Eisenberg D. Three-Dimensional Structure of Membrane and Surface Proteins. *Ann Rev Biochem*. 1984;53:595-623.

23. Chipot C, Maigret B, Rivail J-L. Modeling Amino Acid Side Chains. 1. Determination of Net Atomic Charges from ab initio Self-Consistent-Field Molecular Electrostatic Properties. *J Phys Chem* 1992;96:10276-84.

24. Dwyer D. *Amino Acids: Chemical Properties*. Wiley Encyclopedia of Chemical Biology. 2008:41-51.

25. Parkins DA, Lashmar UT. The Formulation of Biopharmaceutical Products. *Pharm Sci and Tech Today*. 2000;3(4):129-37.

26. Berlin E, Pallansch MJ. Densities of Several Proteins and L-Amino Acids in the Dry State. *Journal of Physical Chemistry*. 1968;72(6):1887-9.

27. Rodante F. Thermal Analysis of Some Alpha-Amino Acids with Similar Structures. *Thermochimica Acta*. 1992;194:197-213.

28. Rodante F. Thermodynamics and Kinetics of Decomposition Processes for Standard Alpha-Amino Acids and Some of their Dipeptides in the Solid State. *Thermochimica Acta*. 1992;200:47-61.
29. Baynes BM, Wang DIC, Trout BL. Role of Arginine in the Stabilization of Proteins Against Aggregation. *Biochemistry*. 2005;44:4919-25.
30. Lam XM, Yang JY, Cleland JL. Antioxidants for Prevention of Methionine Oxidation in Recombinant Monoclonal Antibody HER2. *J Pharm Sci*. 1997;86:1250-5.
31. Wade AM, Tucker HN. Antioxidant Characteristics of L-Histidine. *J Nutritional Biochem*. 1998;9(6):308-15.
32. Ignatova Z, Gierasch LM. Inhibition of Protein Aggregation in vitro and in vivo by a Natural Osmoprotectant. *PNAS*. 2006;103(36):13357-61.
33. Foord RL, Leatherbarrow RJ. Effect of Osmolytes on the Exchange Rates of Backbone Amide Protons in Proteins. *Biochemistry*. 1998;37:2969-78.
34. Rees DC, Robertson AD. Some Thermodynamic Implications for the Thermostability of Proteins. *Protein Science*. 2001;10:1187-94.
35. Taneja S, Ahmad F. Increased Thermal Stability of Proteins in the Presence of Amino Acids. *Biochem J*. 1994;303:147-53.
36. Falconer RJ, Chan C, Hughes K, Munro TP. Stabilization of a Monoclonal Antibody During Purification and Formulation by Addition of Basic Amino Acid Excipients. *J Chem Technol Biotechnol*. 2011;86:942-8.
37. Thakkar SV, Joshi SB, Jones ME, Sathish HA, Bishop SM, Volkin DB, et al. Excipients Differentially Influence the Conformational Stability and Pretransition Dynamics of Two IgG1 Monoclonal Antibodies. *J Pharm Sci*. 2012;101(9):2062-3077.
38. Shiraki K, Kudou M, Fujiwara S, Imanaka T, Takagi M. Biophysical Effect of Amino Acids on the Prevention of Protein Aggregation. *J Biochem*. 2002;132(4):591-5.
39. Santana H, Gonzalez Y, Campana PT, Noda J, Amarantes O, Itry R, et al. Screening for Stability and Compatibility Conditions of Recombinant Human Epidermal Growth Factor for Parenteral Formulation: Effect of pH, Buffers, and Excipients. *Int J Pharm*. 2013.
40. Bolli R, Woodtli K, Bartschi M, Hofferer L, Lerch P. L-Proline Reduces IgG Dimer Content and Enhances the Stability of Intravenous Immunoglobulin (IVIg) Solutions. *Biologics*. 2010;38(150-157).

41. Arakawa T, Tlmasheff SN. Preferential Interactions of Proteins with Solvent Components in Aqueous Amino Acid Solutions. Arch Biochem Biophys. 1983;224(1):169-77.
42. Nakakido M, Kudou M, Arakawa T, Tsumoto K. To be Excluded or to Bind, that is the Question: Arginine Effects on Proteins. Curr Pharm Biotechnol. 2009;10(4):415-420.
43. Golovanov AP, Hautbergue GM, Wilson SA, Lian L-Y. A Simple Method for Improving Protein Solubility and Long-Term Stability. J Am Chem Soc. 2004;126:8933-9.
44. Thakkar SV, Kim JH, Samra HS, Sathish HA, Bishop SM, Joshi SB, et al. Local Dynamics and Their Alteration by Excipients Modulate the Global Conformational Stability of an IgG1 Monoclonal Antibody. J Pharm Sci. 2012;101(12):4444-57.
45. Ito L, Shiraki K, Matsuura T, Okumura M, Hasegawa K, Baba S, et al. High-Resolution X-Ray Analysis Reveals Binding of Arginine to Aromatic Residues of Lysozyme Surface: Implication of Suppression of Protein Aggregation by Arginine. Protein Engineering, Design & Selection. 2011;24(3):269-74.
46. Maeder W, Lieby P, Sebald A, Spycher M, Pedrussio R, Bolli R. Local Tolerance and Stability up to 24 Months of a New 20% Proline-Stabilized Polyclonal Immunoglobulin for Subcutaneous Administration. Biologics. 2011;39:43-9.
47. Chen B-L, Arakawa T. Stabilization of Recombinant Human Keratinocyte Growth Factor by Osmolytes and Salts. J Pharm Sci. 1996;85(4):419-26.
48. Samuel D, Kumar TKS, Ganesh G, Jayaraman G, Yang P-W, Chang M-M, et al. Proline Inhibits Aggregation During Protein Refolding. Protein Science. 2000;9:344-52.
49. Banks DD, Latypov RF, Ketchum RR, Woodard J, Scavezze JL, Siska CC, et al. Native-State Solubility and Transfer Free Energy as Predictive Tools for Selecting Excipients to Include in Protein Formulation Development Studies. J Pharm Sci. 2012;101(8):2720-32.
50. Kumar TKS, Samuel D, Jayaraman G, Srimathi T, Yu C. The Role of Proline in the Prevention of Aggregation During Protein Folding in Vitro. Bio and Mol Bio Int. 1998;46(3):509-17.
51. Maa Y-F, Prestrelski SJ. Biopharmaceutical Powders: Particle Formation and Formulation Considerations. Curr Pharm Biotechnol. 2000;1:283-302.

52. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational Design of Stable Lyophilized Protein Formulations: Some Practical Advice. *Pharm Res.* 1997;14(8):969-75.
53. Izutsu K-i, Kadoya S, Yomota C, Kawanishi T, Yonemochi E, Terada K. Freeze-Drying of Proteins in Glass Solids Formed by Basic Amino Acids and Dicarboxylic Acids. *Chem Pharm Bull.* 2009;57(1):43-8.
54. Chen B, Bautista R, Yu K, Zapata GA, Mulkerrin MG, Chamow SM. Influence of Histidine on the Stability and Physical Properties of a Fully Human Antibody in Aqueous and Solid Forms. *Pharm Res.* 2003;20(12):1952-60.
55. Rajagopal K, Wood J, Tran B, Patapoff TW, Nivaggioli T. Trehalose Limits BSA Aggregation in Spray-Dried Formulations at High Temperatures: Implications in Preparing Polymer Implants for Long-Term Protein Delivery. *J Pharm Sci.* 2013.
56. Zhang MZ, Wen J, Arakawa T, Prestrelski SJ. A New Strategy for Enhancing the Stability of Lyophilized Protein: The Effect of the Reconstitution Medium on Kerinocyte Growth Factor. *Pharm Research.* 1995;12(10):1447-52.
57. Jameel F, Tchessalov S, Bjornson E, Lu X, Besman M, Pikal MJ. Development of Freeze-Dried Biosynthetic Factor VIII: I. A Case Study in the Optimization of Formulation. *Pharm Dev and Tech.* 2009;14(6):687-97.
58. Lale SV, Goyal M, Bansal AK. Development of Lyophilization Cycle and Effect of Excipients on the Stability of Catalase During Lyophilization. *Int J Pharm Investig.* 2011;1(4):214-21.
59. Mattern M, Winter G, Kohnert U, Lee G. Formulation of Proteins in Vacuum-Dried Glasses. II. Process and Storage Stability in Sugar-Free Amino Acid Systems. *Pharmaceutical Development and Technology.* 1999;4(2):199-208.
60. Mattern M, Winter G, Rudolph R, Lee G. Formulation of Proteins in Vacuum-Dried Glasses. I: Improved Vacuum-Drying of Sugars Using Crystallising Amino Acids. *Eur J Pharm Biopharm.* 1997;44:177-85.
61. Tian F, Middaugh CR, Offerdahl T, Munson E, Sane S, Rytting JH. Spectroscopic Evaluation of the Stabilization of Humanized Monoclonal Antibodies in Amino Acid Formulations. *Int J Pharm.* 2007;335:20-31.
62. Tian F, Sane S, Rytting JH. Calorimetric Investigation of Protein/Amino Acid Interactions in the Solid State. *Int J Pharm* 2006;310:175-86.
63. Paik SH, Kim YJ, Han SK, Kim JM, Huh JW, Park YI. Mixture of Three Amino Acids as Stabilizers Replacing Albumin in Lyophilization of New Third Generation Recombinant Factor VIII GreenGene F. *Biotechnol Prog.* 2012;28(6):1517-25.

64. Sane SU, Wong R, Hsu CC. Raman Spectroscopic Characterization of Drying-Induced Structural Changes in a Therapeutic Antibody: Correlatin Structural CHanges with Long-Term Stability. *J Pharm Sci.* 2004;93:1005-18.
65. Li J, Charrerjee K, Medek A, Shalaev E, Zografi G. Acid-Base Characteristics of Bromophenol Blue-Citrate Buffer Systems in the Amorphous State. *Journal of Pharmaceutical Sciences.* 2004;93(3):697-712.
66. Song Y, Schowen RL, Borchardt RT, Topp EM. Effect of 'pH' on the Rate of Asparagine Deamidation in Polymeric Formulations: 'pH' - Rate Profile. *J Pharm Sci.* 2001;90(2).
67. Chang L, Pikal MJ. Mechanims of Protein Stabilization in the Solid State *J Pharm Sci.* 2009;98(9):2886-908.
68. Chang L, Shepherd D, Sun J, Tang X, Pikal MJ. Effect of Sorbitol and Residual Moisture on the Stability of Lyophilized Antibodies: Implications for the Mechanism of Protein Stabilization in the Solid State. *Journal of Pharmaceutical Sciences.* 2005;94(7):1445-55.
69. Cicerone MT, Tellington A, Trost L, Sokolov A. Substantially Improved Stability of Biological Agents in Dried Form: The Rold of Glassy Dynamics in Preservation of Biopharmaceuticals. *BioProcess International.* 2003;V1(1):36-47.
70. Chieng N, Cicerone MT, Zhong Q, Liu M, Pikal MJ. Characterization of Dynamics in Complex Lyophilized Formulations: II. Analysis of Density Variations in Terms of Glass Dyanmics and Comparisons with Global Mobility, Fast Dynamics, and Positron Annihilation Lifetime Spectroscopy (PALS). 2013.
71. Cicerone MT, Soles CL. Fast Dynamics and Stabilization of Proteins: Binary Glasses of Trehalose and Glycerol. *Biophysical Journal.* 2004;86:3836-45.
72. Hora MS, Rana RK, Wilcox CL, Katre NV, Hirtzer P, Wolfe SN, et al. Develoment of a Lyophilized Formulation of Interleukin-2. *Dev Biol Stand.* 1992;74:295-303.

7. Tables

Table 1: Overview of amino acid chemical and physical properties

Amino Acid	Polarity	Solution State Charge(s) at pH 7	pI ^a	Molecular Weight (g/mol)	Hydrophobicity ^b	Potential side chain H bonds ^a	Dipole Moment (D) ^c
Aspartic Acid	Charged	+-	3.0	133	0.417	4	4.33
Glutamic Acid		+-	3.1	147	0.458	4	6.13
Histidine		++	7.6	155	0.548	3	4.04
Lysine		++	9.5	146	0.263	3	9.07
Arginine		++	10.8	174	0.000	7	5.78
Ornithine		++	-	132	-	6	-
Phenylalanine	Hydrophobic	+-	5.9	165	0.951	0	0.29
Tryptophan		+-	5.9	181	0.854	1	1.44
Glycine		+-	6.0	75	0.770	0	-
Valine		+-	6.0	117	0.923	0	0.06
Leucine		+-	6.0	131	0.918	0	0.09
Isoleucine		+-	6.0	131	1.000	0	0.07
Alanine		+-	6.1	89	0.806	0	0.00
Proline		+-	6.3	115	0.678	0	1.47
Cysteine	Polar	+-	5.0	121	0.721	0	1.78
Threonine		+-	5.6	119	0.634	3	1.79
Serine		+-	5.7	105	0.601	3	1.83
Glutamine		+-	5.7	136	0.430	5	3.89
Methionine		+-	5.7	149	0.811	0	1.80
Citrulline		+-	5.9	175	-	4	-

a) NCBI database (19) and Orgovan (18)

b) Eisenberg (22)

c) Chipot (23)

Table 2: Comparison of amino acids on the stabilization of proteins in aqueous formulations.

Amino Acid	Amino Acid (M)	Protein (mg/mL)	Other Excipients	Solution pH**	Stabilization*	Measure of Stability	Assay Used	Key Findings	Ref.
Alanine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+(0.2M), -(0.05M)	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	200 mM concentration resulted in some stabilization, but destabilization at 50 mM.	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	0	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	No significant change.	38
	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	+	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.2	rHEGF (0.10)	100 mM Na-Phosphate Buffer	7.0	+	Thermal stability	Chemical stability was analyzed by RP-HPLC and aggregation was assessed by ELISA assay and SE-HPLC after storage at 50°C	Stability in the presence of an amino acid was compared to stabilization in the presence of only a disaccharide	39
	0.2 - 0.7	Cytochrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Stabilization of native protein structure for hydrophobic amino acid	35
	0.7 - 1.4	BSA (0.036)	0.02 M NaCl	5-8 - 7.9	+	Conformational stability	Densimetry	Induced preferential hydration of protein in solution compared to betaine, thereby providing stabilization	41
	0.7 - 1.4	LYZ (0.15)	0.02 M NaCl	5.8 - 7.9	+	Conformational stability	Densimetry	Induced preferential hydration of protein in solution compared to betaine, thereby providing stabilization	41

Arginine	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	+	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Stabilization at all concentrations.	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Stabilization at all concentrations.	38
	0.1	IgG1 (10)	Buffer type not stated	5.5	0	Thermal stability	Measured aggregation by SE-HPLC	Not efficient in reducing dimer formation.	40
	0.25	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	+	Conformational stability	Intrinsic Trp fluorescence was evaluated as a function of temperature	Stabilized by predominantly influencing conformational stability of solvent exposed regions.	44
	0.25 - 0.7	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	-	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Destabilization native protein structure at all concentrations.	35
	0.3	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	-	Tm	Intrinsic Trp fluorescence over temperature range	Destabilized the protein.	37
Aspartic Acid	0.05 - 0.2	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Stabilization only for heat induced at 50 mM.	38
	0.05 - 0.2	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	-	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Destabilization of the protein.	38
	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	0	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.3	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	-	Tm	Intrinsic Trp fluorescence over temperature range	Destabilized the protein.	37

Glutamic Acid	0.05 - 0.2	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Stabilization only for heat induced at 50 mM.	38
	0.05 - 0.2	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	-	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Destabilization of the protein.	38
	0.3	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	0	Tm	Intrinsic Trp fluorescence over temperature range	No effect on the protein	37
Glutamine	0.05 - 0.2	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Destabilization of the protein.	38
	0.05 - 0.2	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Heat-induced aggregation by storage at 98°C for various times	Stabilization only for heat induced at 50 mM.	38
	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	0	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36

Glycine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	0 (0.2M), - (0.05M)	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	No effect	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	0	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	No effect	38
	0.1	IgG1 (10)	Buffer type not stated	5.3	+	Thermal stability	Measured aggregation by SE-HPLC	Somewhat efficient in reducing dimer formation.	40
	0.2	rHEGF (0.10)	100 mM Na-Phosphate Buffer	7.0	+	Thermal stability	Chemical stability was analyzed by RP-HPLC and aggregation was assessed by ELISA assay and SE-HPLC after storage at 50°C	Stability in the presence of an amino acid was compared to stabilization in the presence of only a disaccharide	39
	0.2 - 3.0	KGF (0.5)	10 mM Na Phosphate	7.0	+	Thermal stability	Stability measured after storage at 37°C by light scattering and anion exchange HPLC.	Stability improved at all concentrations.	47
	0.25 - 0.7	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Stabilization of native protein structure for hydrophobic amino acid	35
	0.3	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	0	Tm	Intrinsic Trp fluorescence over temperature range	No effect on the protein	37
	0.7 - 1.4	BSA (0.036)	0.02 M NaCl	5.8 - 7.9	+	Conformational stability	Densimetry	Induced preferential hydration of protein in solution compared to betaine, thereby providing stabilization	41
	0.7 - 1.4	LYZ (0.15)	0.02 M NaCl	5.8 - 7.9	+	Conformational stability	Densimetry	Induced preferential hydration of protein in solution compared to betaine, thereby providing stabilization	41

Histidine	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	+	T _m	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.09 - 0.17	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	-	T _m	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Destabilization native protein structure at all concentrations.	35
	0.1	IgG1 (10)	Buffer type not stated	5.5	0	Thermal stability	Measured aggregation by SE-HPLC	Not efficient in reducing dimer formation.	40
	0.2	rHEGF (0.10)	100 mM Na-Phosphate Buffer	7.0	+	Thermal stability	Chemical stability was analyzed by RP-HPLC and aggregation was assessed by ELISA assay and SE-HPLC after storage at 50°C	Stability in the presence of an amino acid was compared to stabilization in the presence of only a disaccharide	39
	0.21	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	-	T _m	Intrinsic Trp fluorescence over temperature range	Destabilization of the protein	37
Isoleucine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Best stabilization at 200 mM	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Best stabilization at 200 mM	38
	0.08 - 0.12	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	0	T _m	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	No effect on protein structure	35
	0.12	IgG1 (10)	Buffer type not stated	5.3	+	Thermal stability	Measured aggregation by SE-HPLC	Efficient in reducing dimer formation	40

Leucine	0.05 - 0.10	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	0	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	No effect on protein structure	35
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Best stabilization at 200 mM	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Best stabilization at 200 mM	38
	0.2	rHEGF (0.10)	100 mM Na-Phosphate Buffer	7.0	+	Thermal stability	Chemical stability was analyzed by RP-HPLC and aggregation was assessed by ELISA assay and SE-HPLC after storage at 50°C	Stability in the presence of an amino acid was compared to stabilization in the presence of only a disaccharide	39
Lysine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Improved stability at all concentrations.	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Improved stability at all concentrations.	38
	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	+	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.25 - 0.70	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Stabilization of native protein structure for hydrophobic amino acid	35
	0.3	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	0	Tm	Intrinsic Trp fluorescence over temperature range	No effect on the protein	37

Methionine	0.0145	rhuMAb (21)	4% mannitol, 0.01% polysorbate 20, 0.01% benzethonium chloride	5.0	+	Oxidation	Oxidation measured by HIC.	Oxidation was reduced compared to formulations without methionine	30
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98oC for various times	Best stabilization at 200 mM	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Best stabilization at 200 mM	38
	0.10 - 0.21	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectrscopy	Stabilization of native protein structure for hydrophobic amino acid	35
	0.12	IgG1 (10)	Buffer type not stated	5.3	+	Thermal stability	Measured aggregation by SE-HPLC	Efficient in reducing dimer formation	40
Ornithine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98oC for various times	Improved stability at all concentrations.	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Improved stability at all concentrations.	38
Phenylalanine	0.05 - 0.10	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	0	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectrscopy	No effect on protein structure	35
	0.12	IgG1 (10)	Buffer type not stated	5.3	+	Thermal stability	Measured aggregation by SE-HPLC	Efficient in reducing dimer formation	40

Proline	0 - 0.5	LYZ (1)	0.1 M Tris-HCL	7.2	+	Thermal stability	Aggregation and activity measured by %Transmittance.	Improved activity at higher concentration range. Proposed clusters of amino acid molecules provide a hydrophobic microenvironment for dissolved proteins	48
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98°C for various times	Best stabilization at 200 mM	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Best stabilization at 200 mM	38
	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	0	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.12	IgG1 (10)	Buffer type not stated	5.3	+	Thermal stability	Measured aggregation by SE-HPLC	Efficient in reducing dimer formation. Found to be best stabilizer in this study do to the amphilic properties.	40
	0.2 - 3.0	KGF (0.5)	10 mM Na Phosphate	7.0	+	Thermal stability	Stability measured after storage at 37°C by light scattering and anion exchange HPLC.	Stability improved at all concentrations.	47
	0.25	IgG (20)	0.02 mg/mL polysorbate 80	4.8	+	Thermal stability	Aggregation measured by SE-HPLC	It is proposed that due to proline's amphiphilic nature, it can bind to exposed hydrophobic domains of protein, thereby inhibiting interactions.	46
	0.25 - 0.75	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Stabilization of native protein structure for hydrophobic amino acid	35
	0.3	IgG1 (0.1)	20 mM citrate-phosphate buffer, 0.1 mM NaCl	4.5	0	Tm	Intrinsic Trp fluorescence over temperature range	No effect on the protein	37

Serine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98oC for various times	Stabilization at all concentrations	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	0	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	No Significant stabilization	38
	0.0625	IgG1 (5)	100 mmol/L glycine buffer	3.5	+	Tm	DSC	Side chain played important role in stabilization of protein. Order of Stabilization by amino acids: basic > neutral > acidic	36
	0.1 - 0.2	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Stabilization of native protein structure for hydrophobic amino acid	35
Threonine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98oC for various times	High stabilization at 200 mM	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	0	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	No significant stabilization	38
Valine	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	+	Thermal stability	Heat-induced aggregation by storage at 98oC for various times	Stabilization at all concentrations	38
	0.05 - 0.5	LYZ (0.2)	50 mM Na-Phosphate Buffer	6.5	0	Chemical stability	Amount of protein refolded after aggregation in the presence of 0.8M urea	Best stabilization at 50 mM	38
	0.1 - 0.2	Cytichrome-c (0 - 12000)	0.1 M KCl	3.0	+	Tm	Denaturation in 0.05 M citrate buffer monitored by measuring the change in molar absorption by uv/visible spectroscopy	Stabilization of native protein structure for hydrophobic amino acid	35
	0.12	IgG1 (10)	Buffer type not stated	5.3	+	Thermal stability	Measured aggregation by SE-HPLC	Somewhat efficient in reducing dimer formation.	40

*Stabilization decided based on improved stability (+), minor/no improvement (0), or destabilization of protein (-)

**Values in bold are less than pI of the amino acid

Chapter 3

Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein

Formulations: I. Screening of 15 Amino Acids in Two Model Proteins

Abstract

The purpose of this study was to explore the stabilizing effect of small molecule additives (i.e., amino acids) on the long-term storage stability of lyophilized sucrose-based protein formulations. Two model proteins, recombinant Human Serum Albumin (rHSA) and α -Chymotrypsin (ACT), were formulated with sucrose and 15 amino acid additives, varying in physical and chemical properties. Each formulation was freeze-dried at 1:1:0.3 (w/w) protein:sucrose:amino acid. The total soluble percent aggregation of each formulation was quantified by size exclusion chromatography (SEC) before and after storage for two months at 50°C. Classical thought might suggest that the addition of the amino acids to the sucrose-based protein formulations would destabilize the protein due to a decrease in the system's glass transition temperature (decrease in the structural relaxation time). However, significant improvement in long-term storage stability was observed for almost all formulations at the ratio of amino acids used. Weak correlations were found between the extent of stabilization and the amino acid molar volume; consistent with the mechanism of stabilization potentially being a result of decreased free volume and a resulting suppression of fast dynamics in the amorphous matrix. Multivariable regression analysis further revealed the importance of the amino acid side chain charge and molar volume on the extent of protein stabilization. We conclude that the addition of amino acid additives at a modest level generally improves storage stability, often by more than a factor of two, for lyophilized sucrose-based protein formulations.

1. Introduction

Due to the susceptibility of proteins to both chemical and physical degradation, adequate storage stability is frequently a challenge, resulting in therapeutic protein formulations having an inadequate shelf-life and requiring refrigeration to maintain product quality. Often solution formulations do not have sufficient stability even when refrigerated, so to obtain long-term storage stability, many protein formulations are lyophilized, where stabilization occurs due to the conversion of the product into a solid, providing decreased molecular motion relative to the aqueous solution as well as removing water which can be a reactant(1). However, even if optimal residual moisture is achieved during freeze-drying, degradation processes including aggregation, denaturation, deamination and oxidation can and do still occur(2).

Stability problems in the solid-state are often addressed through alterations in process or formulation, normally with an attempt to increase “native-like” structure in the solid and/or decrease mobility on a pharmaceutically relevant timescale. Generally, to achieve adequate storage stability, excipients such as disaccharides and polymers are added to a formulation to protect the protein from degradation(3-6). The mechanism of stabilization in the dried state is often proposed to be a result of either thermodynamic or kinetic stabilization. A specific type of thermodynamic stabilization, termed the “water substitution” hypothesis, states that the excipient stabilizes the protein by taking the place of water, forming hydrogen bonds with specific sites on the protein. The excipient thereby protects the native protein structure and provides conformational stabilization in the solid-state via increasing the free energy of unfolding. On the other hand, kinetic

stabilization, also referred to as the “glass dynamics” hypothesis, states that the stabilizer immobilizes the protein molecules in a rigid matrix, providing spatial separation of potential reactive sites, thereby stabilizing regardless of the thermodynamics of unfolding(7).

Kinetic stabilization is often discussed in terms of the glass transition temperature (T_g , a parameter correlated with the onset of viscous flow and a measure of global mobility) where it has often been suggested that at storage temperatures below the formulation T_g , motions will be greatly hindered, thus suppressing degradation reactions. Most studies show, at best, weak correlations between T_g and stability (for systems stored at temperatures well below their T_g), and often a better measure of global mobility is with structural relaxation time, obtained from calorimetry measurements(8). The timescale of global mobility is comparable to that of degradation (100 seconds at T_g and slower within the glass); however, the correlation between stability and structural relaxation times is often poor(3, 9).

Recently, more local motions within the glass, presumably resulting from intra-molecular reorientations occurring on a much shorter timescale (microsecond to nanoseconds), have been suggested to be controlling stability in systems well below T_g (10). These fast motions are highly correlated to free-volume and according to the classic view of free volume, the extent of mobility is related to the amount of available open space around a molecule(11). Such free volume nominally reflects that volume accessible to a given molecule without concerted movement of its neighbors. That is, molecular motion

cannot take place unless there are neighboring “open” spaces for a molecule to occupy (12). Therefore, the amount of local motion will increase with the free volume accessible to the molecules (13) and in theory, filling in the “open” spaces around each molecule should restrict motions, thereby leading to a suppression of the fast-dynamics. The validity of this idea is supported by the observation that the addition of low levels of small molecule additives, glycerol and sorbitol, to disaccharide-based protein formulations was correlated with a suppression of fast dynamics and increased stability(14, 15).

Normally, these types of “small molecules” that remain amorphous are avoided in solid-state formulations since they have low T_g 's and are classically expected to destabilize the product by lowering the T_g of the system and decreasing structural relaxation time(14, 16). In addition, a low T_g often results in a low collapse temperature, making control of the freeze-drying process more difficult. However, even though plasticization of global motions occurs (i.e., increased cooperative motions, decreased structural relaxation time), the small molecule additives anti-plasticize the fast-dynamics (i.e., decrease non-cooperative motions, suppressed local motions), which results in improved stability(17).

Additionally, other studies have investigated the impact of other types of “small molecules” on long-term stability of lyophilized proteins. For example water, which is often minimized in solid-state formulations, was found to improve the chemical and physical stability of several disaccharide-based antibody formulations(16, 18). Histidine,

commonly used as a buffering agent, was found to improve the stability of an enzyme when added to freeze-dried formulations at higher than normal concentrations(19). Thus, the addition of the small molecule additives may be critical for optimal storage stability; however, it is not well understood what properties of the small molecule additive (e.g., molecule size, charge state, etc.) are important to the suppression of fast dynamics and stabilization. Once better understood, small molecule additives could be promising candidates to use as stabilizers and potentially be one method to obtain room temperature stability.

The primary objective of this study was to investigate the stabilizing effects of other small molecule additives on the long-term storage stability of freeze-dried protein formulations in order to better understand the stabilization effect provided by small molecules. A series of amino acids were chosen in order to establish if any properties related to the additives chemical nature, charge, or size were correlated with stabilization. Two model proteins, recombinant Human Serum Albumin (rHSA) and α -Chymotrypsin (ACT), were formulated with sucrose along with 15 amino acid additives. All formulations were prepared at one amino acid concentration in order to efficiently investigate the impact of stabilization by the amino acid additive on the already relatively stable sucrose-based protein formulations.

2. Materials and Methods

2.1 Materials

Ultra-pure recombinant human serum albumin (rHSA) was purchased from Albumin Biosciences (Huntsville, AL). The material was recombinant protein with certified to have purity greater than 99% as determined by SDS-PAGE and IEF. α -Chymotrypsin (ACT) was purchased from MP Biomedicals (Solon, OH) as lyophilized powder, essentially salt free and greater than 94% purity. The phosphate buffers, sucrose (SUC), sorbitol and all amino acids (Table 1) were purchased from Sigma (St. Louis, MO). All excipients were of the highest grade available and used without further purification. All amino acids were of the neutral form and salt free.

2.2 Sample Preparation

Before freeze-drying, rHSA (MW = 66.4 kDa, pI = 4.7) and ACT (MW = 25.6 kDa, pI = 8.7) were dialyzed against 2mM sodium phosphate buffer at pH 7 using a dialysis membrane with MWCO: 6-8,000 kDa (Spectrum Laboratories, Dominguez, CA). After dialysis, protein concentrations were determined by measuring UV absorbance at 280 nm (Cary 50 Bio, Varian). The final concentrations of rHSA and ACT were 5 mg/mL and 10 mg/mL, respectively. Separately, sucrose (100 mg/mL), sorbitol (50 mg/mL), and each amino acid (50 mg/mL) were dissolved into 2mM phosphate buffer at pH 7.¹

¹ The pH of each bulk solution was adjusted to pH 7 as needed. For the acidic amino acids (aspartic acid and glutamic acid) 1M NaOH was added to the bulk solutions. For the basic amino acids (histidine, lysine, and arginine) 1M HCl was added to bulk solutions. The amount of salt formed as a result of titrating to pH 7 was four orders of

Solutions of 1:1:0.3 rHSA:sucrose:additive and 1:1:0.3 ACT:sucrose:additive were prepared from the bulk solutions. Before freeze-drying, final solutions were filtered through 0.22 μm filters and 1mL of the solution was filled into 5 cc glass tubing vials and semi-stoppered with Daikyo Flurotech stoppers (West Pharmaceutical, Lionville, PA), which are low moisture release stoppers.

2.3 Lyophilization

Solutions were lyophilized in a Durastop Freeze dryer (FTS, SP Industries, Stone Ridge, New York). Thermocouples were placed in the bottom center of at least four vials per batch. After thermal equilibration at -5°C , samples were cooled to -42°C at a rate of $1^{\circ}\text{C}/\text{min}$ during the freezing step. Primary drying was carried out at a shelf temperature of -32°C and a chamber pressure of 80 mTorr for at least 20 hours, and product temperature during primary drying was maintained well below collapse temperatures (discussed in section 3.1). The end of primary drying was taken as the time all product temperatures approached the shelf temperature, which indicates the end of primary drying in the thermocouple vials. Before moving to secondary drying, an additional “soak” time of 4 hours was used to ensure complete ice removal in all vials. For secondary drying, the temperature was increased to 40°C at $1^{\circ}\text{C}/\text{min}$ and held for 6 hours. Upon completion, vials were stoppered under nitrogen and partial vacuum (650 – 700 Torr) and crimped with aluminum seals. All freeze-dried products dried with retention of structure and showed no visible signs of collapse. Samples were confirmed

magnitude less than the ionized form. Therefore, the salt formed should essentially have no effect on the T_g' or impact the stability results.

to be amorphous by polarizing light microscopy, as judged by no birefringence. Water content of selected formulations was between 0.1-0.3% (w/w) as determined by Karl-Fischer moisture titration (756 Coulometric Titrator, Metrohm, Riverview, FL).

2.4 Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (Q1000) from TA Instruments (New Castle, DE) was used to determine the glass transition temperature (T_g), the change in heat capacity (ΔC_p) at T_g , and the T_g' (T_g of the maximally concentrated freeze concentrate). For T_g' measurements, solution volumes of 10 μ L were hermetically sealed in aluminum pans. Samples were equilibrated at -70°C and ramped at $10^\circ\text{C}/\text{min}$ to 0°C . T_g' values are shown in Table 1.

Modulated DSC was used to determine T_g and ΔC_p for solid-state formulations, using a ramp rate of $1^\circ\text{C}/\text{min}$, amplitude of $\pm 0.82^\circ\text{C}$ and period of 80 seconds. Samples were prepared by gently breaking up the lyophilized cake and compacting 5-10 mg of the powder into the bottom of an aluminum pan, in the form of a thin disk. Samples were hermetically sealed in the pan for analysis. All samples were prepared at low humidity conditions ($< 3\% \text{RH}$) in a dry bag continuously purged with dry air or nitrogen. The values of T_g' and T_g were taken as the midpoint of the transition from the DSC thermogram.

2.5 Density Measurements by Gas Pycnometer

Densities of selected crystalline amino acids were measured using an AccuPyc 1330

helium pycnometer (Micrometrics, Norcross, GA) at room temperature (23°C) in order to calculate the molar volume of the pure amino acids. All procedures were carried out in a glove bag with controlled humidity (< 3% RH) using a dry 1 mL aluminum sample cup. Calibration was done using an iron sphere standard of known volume. The density measurements were carried out as previously reported(20). Briefly, 100 mg of sample was gently packed into the aluminum cup and transferred to the pycnometer for measurement. Each sample was measured 20 times, with 99 purges and the obtained density value was the average of the measurements. Three independent sample measurements were performed for each formulation and the standard error of the mean was about 0.2%. The measured crystalline densities of the amino acids were comparable to literature values(21). All other amino acid crystalline densities used to calculate molar volumes were obtained from the same literature reference(21).

2.6 Stability Studies

Long-term storage stability was evaluated by determining the percent total soluble protein aggregates after processing (time zero) and after two months storage at 50°C. The percentage of soluble aggregate was determined by size exclusion chromatography (SEC) using a TSKgel G2000SW_{XL} column (7.8 mmID x 30 cm, 5 µm, Tosoh Bioscience). Freeze-dried samples were reconstituted with purified water, appropriately diluted and filtered, before injection onto the column. After reconstitution, all vials were

visually clear.² The rHSA and ACT peaks were detected by UV absorbance at 254 and 280 nm, respectively. For rHSA formulations, a mobile phase of 50 mM potassium phosphate buffer at pH 6.5 with 200 mM Na₂SO₄, a flow rate of 1 mL/min and column temperature of 30°C was used. For ACT formulations, a mobile phase of 50 mM sodium phosphate buffer at pH 7 with 50 mM NaCl, a flow rate of 0.5 mL/min and column temperature of 25°C was used. In general, to check for reproducibility two replicate samples were assayed at time zero and two months. The mean difference between replicate samples was 0.2% for rHSA and 0.04% for ACT aggregation analysis.

A rate constant for aggregation was not determined due to the large number of samples; however, previous studies on several similar formulations showed storage stability follows square root of time kinetics(3, 22). To compare stability between formulations, a stabilization ratio (R_s) was calculated. The amount of measured total soluble aggregation after freeze-drying (in-process aggregation) was subtracted from the amount of measured total soluble aggregation after two months storage, to obtain the total percent aggregation during storage (ΔA) for each formulation. In order to evaluate the extent of stabilization with an additive (ΔA^x), compared to the base protein-sucrose formulation (ΔA^0), a stabilization ratio (R_s) for each formulation, as shown in Equation 1.

$$R_s = \frac{\Delta A^0 - \Delta A^x}{\Delta A^0} \quad (1)$$

² Cysteine was used as one of the selected amino acids. However, upon reconstitution, the cysteine precipitated out of solution. For this reason, no cysteine formulations could be loaded onto the SEC for aggregation analysis.

Here, ΔA^0 is the increase in aggregation during storage for the base-sucrose protein system without any additive and ΔA^x is the increase in aggregation during storage for the system with additive. A larger stabilization ratio (R_s) indicates better stabilization with the selected amino acid additive (A^x) compared to the sucrose-based protein formulation containing no additive (A^0). Perfect stabilization would correspond to $R_s = 1$, no additional stabilization by the additive would mean $R_s = 0$, and destabilization by the additive would mean a negative value.

2.7 Statistical Analysis

For the accelerated stability study, the statistical analysis was performed using Microsoft Excel (Version 14.3.4, USA). A significance level was considered at a p-value < 0.05 (95% confidence level).

To evaluate the contribution of each amino acid variable on the stability a Pearson's correlation was evaluated and multivariable regression analysis was conducted with XLSTAT (Version 2013.3.01, Addinsoft, New York, NY). The Pearson's correlation coefficients were considered significantly correlated at p-value < 0.05 (95% confidence level), and variables in the multivariable regression analysis were considered significant at p-value < 0.20 (80% confidence level). Before analysis, the data was transformed to similar ranges and centered around the mean, which helped with analysis interpretation. Multivariable analysis was attempted using non-correlated variables from Table 2, as determined from the Pearson's coefficients. Models with both main effects and

interaction effects were evaluated to determine the variables that contributed significantly to the stabilization ratio analysis for each protein formulation series.

3. Results and Discussion

3.1 Evaluation of Formulation T_g' Values

T_g' is measured when developing a freeze-drying process in order to set an appropriate shelf-temperature and therefore insure that the product temperature does not exceed the collapse temperature, which is typically a few degrees higher than the corresponding T_g' . For these experiments, T_g' values of 0.05:1 w:w amino acid:SUC solutions (without protein) were determined by DSC (Table 1). The T_g' values for the amino acid formulations differ by only 5°C, with the lysine:SUC formulation having the lowest T_g' at -37°C.

T_g' for solutions of only 1:1 ACT:SUC and 1:1 rHSA:SUC were also evaluated by DSC. These systems showed relatively high T_g' values (-22°C for 1:1 rHSA:SUC and -20°C for 1:1 ACT:SUC). Since the T_g' for the amino acid:SUC formulations were all near the T_g' of pure sucrose (-35°C(23)), the impact of the amino acid at this level on the T_g' of a predominately sucrose system is minimal. Therefore, the T_g' for the three component system of 1:1:0.3 protein:SUC:amino acid systems should all be close to the value for the pure protein:SUC formulations (around -22°C or -20°C). A shelf temperature of -32°C was used for the lyophilization of all formulations in this study, which resulted in products with no visible signs of collapse. The maximum product temperature recorded

during primary drying was -33°C and -32°C for 1:1:0.5 ACT:SUC:lysine formulations and 1:1:0.5 rHSA:SUC:lysine formulations, respectively, which was below the estimated collapse temperature of the formulations.

3.2 Evaluation of Formulation T_g Values

The glass transition temperatures (T_g) for pure amino acids were estimated using literature melting temperatures(24) and assuming the ratio $T_g/T_m = 0.8$ (25) (not shown). T_g was measured directly for the 1:1 ACT:SUC and 1:1 rHSA:SUC formulations. The mid-point T_g value for the 1:1 ACT:SUC formulation is 66°C ($\Delta C_p = 0.71 \text{ J/g/}^\circ\text{C}$) and the T_g is 100°C ($\Delta C_p = 0.36 \text{ J/g/}^\circ\text{C}$) for the 1:1 rHSA:SUC formulation. The ACT:SUC formulation has a larger change in heat capacity and a lower T_g than the rHSA:SUC formulation. The reason for these differences is not obvious, however, as will be discussed later, the majority of the ACT-sucrose formulations have better stability than the rHSA-sucrose formulations indicating that a high T_g is not necessarily representative of better stability.

Using the Fox equation(26), the T_g for the 1:1:0.3 protein:sucrose:amino acid formulations was estimated (not shown). All rHSA formulations at 1:1:0.3 have calculated T_g values much higher than the storage temperature of 50°C and therefore should remain in the glassy state throughout the stability study. While all estimated T_g values for the ACT formulations were also greater than 50°C, the T_g estimates for several formulations are relatively close to 50°C. However, there was no visible

shrinkage of the cakes during storage, providing evidence that the samples remained below the T_g values during the two months stability study.

3.3 Stability Results

All formulations were confirmed amorphous after storage by polarized light microscopy. For several representative formulations, the water content after storage was determined by Karl-Fischer moisture titration to be between 0.2-0.6% (w/w), indicating there was insignificant water transfer from the stopper to the cake over two months at 50°C. For this reason, it can be concluded that the difference in protein stability for the formulations studied was not a result of differences in residual moisture. For these formulations, soluble protein aggregation was measured due to the ease of precise characterization by HPLC.

3.3.1 ACT Stability Results

Before freeze-drying pure α -Chymotrypsin (ACT) had less than 0.1% soluble aggregation. After freeze-drying, the α -Chymotrypsin (ACT) formulations also had less than 0.1% aggregation, indicating minimal loss in stability during processing. Most of the amino acids enhanced the stability of the ACT:Sucrose formulation (Figure 1, gray bars). Aspartic acid was the only amino acid to significantly destabilize ACT, with almost twice the level of aggregation as in the formulation without aspartic acid. One might speculate that this destabilization was due to the fact that at least in solution, aspartic acid has a negatively charged side chain, which could be destabilizing to ACT. However, glutamic acid also has a negatively charged side chain in solution, and this

amino acid enhanced the stability of ACT. Therefore, the destabilization must arise from other causes, the nature of which is obscure. All of the other amino acids studied improved the physical stability of ACT (significant at $p\text{-value} < 0.05$). Serine improved the stability of ACT to the greatest extent, with a five fold improvement in stability.

3.3.2 rHSA Stability Results

The soluble aggregation for recombinant Human Serum Albumin (rHSA) before freeze-drying was close to 1% and after freeze-drying, the rHSA formulations had an average of 1.8% soluble aggregate, indicating some stability loss during processing. There was essentially the same level of aggregate for all formulations, indicating the degradation after freeze-drying was not due to the addition of the amino acids.

The results of the two month stability study at 50°C for the 1:1:0.3 rHSA:SUC:Amino acid formulations are shown in Figure 1 (stripped bars), where almost all amino acids stabilize rHSA compared to the formulation without any amino acid additive. The addition of phenylalanine provided no additional stability and actually resulted in slightly decreased stability; however, the difference in stability from the base sucrose formulation was within the combined experimental errors. All of the other formulations containing amino acids did significantly stabilize the rHSA (significant at $p\text{-value} < 0.05$). Formulations with valine, threonine, arginine and citrulline were almost four times more stable than the formulation without any amino acid. From Figure 1, there is no obvious correlation between stabilization, side chain group or molar volume.

3.4 Correlation of Stability with Amino Acid Properties

The amino acids studied were divided into four main categories based on the side chain properties of the molecules in solution(27) at pH 7. The classes are as follows: 1) Hydrophobic, 2) Uncharged Polar, 3) Positively Charged, and 4) Negatively Charged, (Table 2). The relationship between the amino acid side chain properties and the extent of stabilization by the amino acids for both protein formulations was explored (Figure 2). We note that all amino acids stabilize rHSA better than ACT at the concentration of 1:1:0.3 protein:SUC:amino acid. For ACT, the improvement in stabilization is similar for the hydrophobic and uncharged polar amino acids. There is significantly greater stabilization ($p\text{-value} < 0.05$) resulting from the positively charged amino acids (arginine, lysine and histidine). For the negatively charged amino acids, aspartic acid was excluded from the analysis due to the significant (atypical) destabilization of ACT (shown in Figure 1). For rHSA, the four amino acid groups stabilize to a similar extent, where polar uncharged and positively charged amino acids have the greatest improvement in stability. From Figure 2 it is apparent that both the extent of stabilization and qualitative trends are somewhat protein specific.

It is important to note that there is no correlation between the estimated glass transition values and the extent of stabilization (analysis not shown). This indicates that although the protein-sucrose glass transition temperature is decreased with the addition of amino acids, this does not produce a decrease in stability and shelf-life of the protein formulations.

3.4.1 Correlation of Stability with Molar Volume

If stability is related to the amount of available free volume of the amorphous matrix, then the size of the amino acid and its “fit” into the protein:sucrose matrix might be expected to be directly correlated to the stabilization ratio. To best assess the “fit” of the amino acid in the amorphous matrix, the molar volume was determined; where molar volume is considered to be an adequate representation of the molecule size. Densities of pure crystalline aspartic acid, lysine, alanine, threonine, serine, and methionine were measured experimentally using a He pycnometer. All other density values for the amino acids were determined from the literature(24). The molar volume of each amino acid was calculated as the ratio of molecular weight to density (Table 2). It is noted that the molar volume is calculated from the crystalline density of the amino acid and is therefore not entirely representative of the molar volume for the amino acid in the amorphous state. However, the qualitative trends in density should still be the same.

The molar volume of each amino acid was compared to the stabilization ratio for each group of amino acid. For the amino acids with charged side chains (both positively and negatively charged), there is a correlation between molar volume and stabilization ratio for both ACT ($R^2 = 0.579$) and rHSA ($R^2 = 0.820$) (data not shown). The observation indicates that larger charged amino acids stabilize better than smaller charged amino acids, suggesting the “fit” of the amino acid into the amorphous matrix may be better for larger, charged molecules. It is not clear if this correlation is a result of the charge effect at pH 7 or the effect of the molar volume size. The positively charged amino acids are both larger and more stabilizing than the negatively charged amino acids, therefore,

what is revealed is not necessarily the size effect on stabilization but the difference in stabilization between the positively and negatively charged amino acids. Multiple regression analysis was used to further investigate this “apparent” correlation. For amino acids with hydrophobic side chains and uncharged polar side chains, there is not a significant correlation between the size of the amino acid and the extent of stabilization for both proteins studied.

3.5 Multiple Regression Analysis

An attempt to relate various amino acid chemical and physical characteristics (molar volume, side chain charge (i.e., polarity), isoelectric point (pI), hydrophobicity, dipole moment, side chain hydrogen bond potential and side chain flexibility (24, 28, 29)(Table 2)) with the stabilization ratio of the amino acid formulations was attempted using multiple regression analysis. Before the regression analysis was conducted, the variables of interest were tested for collinearity, which occurs when two or more variables are correlated to a high extent such that they provide the same information about the variations in the dependent values. Collinearity may result in uncertainty of the estimated coefficients in the model and large standard errors. To test for the presence of collinearity, a Pearson’s correlation coefficient for each possible interaction variable was calculated, shown in Table 3. Side chain charge (i.e., polarity) and pI are highly correlated ($R^2 = 0.946$) as well as molar volume and side chain flexibility ($R^2 = 0.696$). Dipole moment, potential hydrogen bonds and hydrophobicity are all highly correlated, therefore, representative of similar properties. Hydrophobicity is negatively correlated

with both potential hydrogen bonds ($R^2 = -0.911$) and dipole moment ($R^2 = -0.878$), while potential hydrogen bonds and dipole moment are positively correlated ($R^2 = 0.738$). Dipole moment is also significantly correlated to side chain flexibility ($R^2 = 0.663$), where higher dipole moments mean greater side chain flexibility. The high correlations indicate a large degree of similarity between the amino acid properties listed in Table 2 and choosing one variable allows for potential interpretation of the other correlated variables.

In an attempt to understand the relationship between amino acid stabilization and amino acid properties, and to avoid collinearity, three variables were chosen in the multiple regression analysis of the stability data for both ACT and rHSA formulations. The variables molar volume, side chain charge (i.e., polarity) and hydrophilicity were selected as they provided an adequate representation of all the variables listed in Table 2, while avoiding potential collinearity issues. Molar volume was chosen over side chain flexibility, due to the continuous nature of the values. Also, as discussed earlier, there is an indication that the size of the additive may be related to the improved stability. The side chain charge (i.e., polarity) was chosen over the isoelectric point, since the charge represents the state of the additive at the pH of interest (in this case pH 7). Potential side chain hydrogen bonds were not chosen due to the non-continuous nature of the data. Finally, hydrophobicity was chosen as the third variable, which is an average of several hydrophobicity scales (at pH 7), representative of the side chain of each additive, compared with dipole moment, which is from a single analysis.

The results of the multivariable analysis for the ACT stability data and rHSA stability data are shown in Table 4 and Table 5, respectively. Aspartic acid was not included in the ACT analysis due to atypical destabilization (Figure 1). The variables molar volume and side chain charge (polarity) are significant for both protein formulations at a p-value < 0.20 (80% confidence interval). For ACT, the average experimental and calculated R_s values were both 0.283. The standard deviation of the mean for the experimental R_s values was ± 0.115 while the standard deviation of the mean for the calculated R_s values was ± 0.064 . The average relative deviation of the calculated R_s from the experimental R_s was 0.005. This indicates that the accuracy of the model is similar to the accuracy of the data, and therefore, the model in Table 4 is a good predictor of R_s for ACT. For the rHSA data, the average calculated R_s value is 0.512, which is slightly larger than the average experimental R_s value of 0.492. The standard deviation of the mean in the experimental R_s values is ± 0.223 while the standard error of the mean in the calculated R_s values is ± 0.136 . The average relative deviation of the calculated R_s from the experimental R_s was -0.426. The negative average relative deviation indicates that the model underpredicts the stabilization ratio, however, this value is still in the same range as the experimental standard deviation (± 0.223), indicating the model in Table 5 is still a fair predictor of stability. All rHSA errors are larger than the ACT errors, which is most likely due to the larger spread in rHSA stability data versus the ACT stability data and most likely the explanation for the poorer model accuracy compared with the ATC model. However, the model for rHSA indicates that both charge and molar volume significantly impact the stabilization ratio.

Analysis of hydrophobicity and interactions between the major variables (data not shown) indicate that these effects add nothing of value to the model. That is, when added to the model, hydrophobicity and “interactions effects” cancel each other out. Both ACT and rHSA results indicate improved stability for positively charged and smaller molar volume additives. These results support the conclusions made in section 3.4, where the larger, positively charged amino acids stabilized to a greater extent than the negatively charged amino acids, which likely means that it was not the size but rather the sign of the charge that was important.

It is important to emphasize that the properties in Table 2 are based on the solution state of the amino acid, and any lack of correlation with the solid-state stability may be due to the change in properties resulting from going to the solution to the solid-state during freeze-drying. For example, it has been documented that the pKa and the polarity of a small molecule changes in the solid state(30).

3.6 Stabilization and Ionic Interaction

Given the experimental observations and statistical analysis, it is not obvious how electrostatic interactions between the protein's net charge and the amino acid's net charge play a major role in the stabilization. At pH 7, rHSA is negatively charged in solution, which might suggest ionic interactions between protein and stabilizer could explain the increased stabilization by the positively charged amino acids (lysine, arginine, histidine). By the same logic, ACT is positively charged in solution at pH 7 and the negatively charged amino acids (aspartic acid, glutamic acid) should greatly improve

stability, which is not the case. The ACT and rHSA stabilities have high to modest correlations with side chain charge of each amino acid (discussed earlier), which is suggestive of ionic interaction involvement. These trends might possibly be attributed to the different net charge on the protein in the solid than in dilute solution; however, more likely it is the result of the surface charge of a protein being not uniformly distributed over the surface but rather being present in discrete regions of different charge, where the amino acid could potentially be interacting with charged patches on the protein, thereby providing stabilization. Therefore, the ionic interactions may be important to the mechanism of stabilization by the amino acid; however, the interactions seem to be more complex than interactions between net charges on the amino acid and protein. This mechanism has been documented for the solution state where arginine suppressed aggregation by interacting with the surface of lysozyme(31). It is possible that the stabilization of the proteins is a result of the protein surface charge and amino acid interactions.

3.7 Comparison with Sorbitol

Previously sorbitol, investigated in 1:1:X IgG1:Sucrose:Sorbitol formulations at various levels, was found to stabilize the proteins after storage at 50°C for 2 months. At the ratio of 1:1:0.3, IgG1:SUC:Sorbitol, it was found that sorbitol stabilized by more than a factor of two relative to the formulation without sorbitol(16). Figure 3 compares the percent stabilization by sorbitol at 1:1:0.3 for rHSA:SUC:Sorbitol, ACT:SUC:Sorbitol and IgG1:SUC:Sorbitol formulations. Sorbitol stabilizes both rHSA and IgG1 but does not

stabilize ACT, at least at this concentration. It appears that the extent of stabilization is quite sensitive to the specific protein being investigated as well as the type of stabilizer, as suggested by data discussed earlier.

4. Conclusions

This study demonstrates the stabilizing effect of low levels of a number of amino acid additives on the long-term storage stability of model freeze-dried disaccharide-based protein formulations (rHSA:SUC and ACT:SUC). Clearly, stabilization by small molecules is not restricted to just polyols (i.e., glycerol, sorbitol) but rather can be obtained from most amino acids. Both rHSA and ACT data show modest correlations between the molar volume and the stabilization ratio for the charged amino acids. This suggests that “the fit” of amino acids into the “free volume holes” of the amorphous formulations matrix, is important in suppressing dynamics on a fast timescale, thereby leading to improved stability. Further statistical analysis further revealed the importance of molar volume and side chain charge (polarity). Interestingly, almost all of the amino acids studied showed an improvement in stability for both protein formulations (in some cases stability is improved by a factor of five). However, based on the results of the sorbitol study, it appears that stabilization may be protein specific. The small molecule additives have the potential for significantly improving stability for disaccharide protein formulations, facilitating development of room temperature stable products.

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6. References

1. Maa Y-F, Prestrelski SJ. Biopharmaceutical Powders: Particle Formation and Formulation Considerations. *Curr Pharm Biotechnol*. 2000;1:283-302.
2. Zhang L, Sinha S, Topp EM. Protein Conformation and Reactivity in Amorphous Solids. In: Jameel F, Hershenson S, editors. *Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals*. Hoboken, New Jersey: John Wiley & Sons, Inc.; 2010. p. 493-506.
3. Wang B, Tchessalov S, Cicerone MT, Warne NW, Pikal MJ. Impact of Sucrose Level on Storage Stability of Proteins in Freeze-Dried Solids: II. Correlation of Aggregation Rate with Protein Structure and Molecular Mobility. *Journal of Pharmaceutical Sciences*. 2008;98(9):1-22.
4. Wang B, Tchessalov S, Warne NW, Pikal MJ. Impact of Sucrose Level on Storage Stability of Proteins in Freeze-Dried Solids: I. Correlation of Protein-Sugar Interaction with Native Structure Preservation. *J Pharm Sci*. 2009;98:3131-44.
5. Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. Factors Affecting Short-Term and Long-Term Stabilities of Proteins. *Advanced Drug Delivery Reviews*. 2001;46:307-26.
6. Luthra S, Obert J-P, Kalonia DS, Pikal MJ. Impact of Critical Process and Formulation Parameters Affecting In-Process Stability of Lactate Dehydrogenase During the Secondary Drying Stage of Lyophilization: a Mini Freeze Dryer Study. *J Pharm Sci*. 2007;96(9):2242-50.
7. Chang L, Pikal MJ. Mechanisms of Protein Stabilization in the Solid State *J Pharm Sci*. 2009;98(9):2886-908.

8. Yoshioka S, Aso Y. Correlations Between Molecular Mobility and Chemical Stability During Storage of Amorphous Pharmaceuticals. *Journal of Pharmaceutical Sciences*. 2006;96(5):960-81.
9. Pikal MJ, Rigsbee D, Roy ML, Galreadth D, Kovach KJ, Wang B, et al. Solid State Chemistry II: The Correlation of Storage Stability of Freeze-Dried Human Growth Hormone (hGH) with Structure and Dynamics in the Glassy Solid. *Journal of Pharmaceutical Sciences*. 2008;97(12):5106-21.
10. Cicerone MT, Tellington A, Trost L, Sokolov A. Substantially Improved Stability of Biological Agents in Dried Form: The Rold of Glassy Dynamics in Preservation of Biopharmaceuticals. *BioProcess International*. 2003;V1(1):36-47.
11. Cohen MH, Turnbull D. Molecular Transport in Liquids andGlasses. *J Chem Physics*. 1959;31(5):1164-9.
12. Sperling LH. *Introduction to Physical Polymer Sciences*. New York: John Wiley & Sons, Inc.; 1992.
13. Simmons DS, Cicerone MT, Zhong Q, Tyagi M, Douglas JF. Generalized Localization Model of Relaxation in Glass-Forming Liquids. *Soft Matter*. 2012;8:11455-61.
14. Chieng N, Cicerone MT, Zhong Q, Liu M, Pikal MJ. Characterization of Dynamics in Complex Lyophilized Formulations: II. Analysis of Density Variations in Terms of Glass Dyamics and Comparisons with Global Mobility, Fast Dynamics, and Positron Annihilation Lifetime Spectroscopy (PALS). 2013.
15. Cicerone MT, Soles CL, Chowdhuri Z, Pikal MJ, Chang L. Fast Dynamics as a Diagnostic for Excipients in Preservation of Dried Proteins. *American Pharmaceutical Review*.22-7.

16. Chang L, Shepherd D, Sun J, Tang X, Pikal MJ. Effect of Sorbitol and Residual Moisture on the Stability of Lyophilized Antibodies: Implications for the Mechanism of Protein Stabilization in the Solid State. *Journal of Pharmaceutical Sciences*. 2005;94(7):1445-55.
17. Cicerone MT, Soles CL. Fast Dynamics and Stabilization of Proteins: Binary Glasses of Trehalose and Glycerol. *Biophysical Journal*. 2004;86:3836-45.
18. Breen ED, Curley JG, Overcashier DE, Hsu CC, Shire SJ. Effect of Moisture on the Stability of a Lyophilized Humanized Monoclonal ANTibody Formulation. *Pharm Res*. 2001;18(9):1345-53.
19. Al-Hussein A, Gieseler H. Investigation of Histidine Stabilizing Effects on LDH During Freeze-Drying. *J Pharm Sci*. 2012;102(3):813-26.
20. Kikuchi T, Wang BS, Pikal MJ. High-Precision Absolute (True) Density Measurements on Hygroscopic Powders by Gas Pycnometry: Application to Determining Effects of Formulation and Process on Free Volume of Lyophilized Products. *Journal of Pharmaceutical Sciences*. 2011:1-7.
21. Berlin E, Pallansch MJ. Densities of Several Proteins and L-Amino Acids in the Dry State. *Journal of Physicl Chemistry*. 1968;72(6):1887-9.
22. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang X, et al. Mechanism of Protein Stabilization by Sugars During Freeze-Drying and Storage: Native Structure Preservation, Specific Interaction, and/or Immobilized in a Glassy Matrix? *Journal of Pharmaceutical Sciences*. 2005;94(7):1427-44.
23. Wang W. Lyophilization and Development of Solid Protein Pharmaceuticals. *Int J Pharm*. 2000;203:1-60.

24. Dwyer D. Amino Acids: Chemical Properties. Wiley Encyclopedia of Chemical Biology. 2008;41-51.
25. Bhugra C, Pikal MJ. Role of Thermodynamic, Molecular, and Kinetic Factors in Crystallization From the Amorphous State. Journal of Pharmaceutical Sciences. 2008;97(4):1329-49.
26. Donth E-J. Relaxation and Thermodynamics in Polymers: Glass Transition. Berlin: Akademie Verlag; 1992. 355 p.
27. Creighton TE. Proteins: Structures and Molecular Properties. 2nd ed. New York: W. H. Freeman and Company; 1993.
28. Orgovan G, Noszal B. The Complete Microspeciation of Arginine and Citrulline. Journal of Pharmaceutical and Biomedical Analysis. 2011;54:965-71.
29. NCBI. Amino Acid Explorer: United States Government; 2009.
30. Li J, Chatterjee K, Medek A, Shalae E, Zografi G. Acid-Base Characteristics of Bromophenol Blue-Citrate Buffer Systems in the Amorphous State. Journal of Pharmaceutical Sciences. 2004;93(3):697-712.
31. Ito L, Shiraki K, Matsuura T, Okumura M, Hasegawa K, Baba S, et al. High-Resolution X-Ray Analysis Reveals Binding of Arginine to Aromatic Residues of Lysozyme Surface: Implication of Suppression of Protein Aggregation by Arginine. Protein Engineering, Design & Selection. 2011;24(3):269-74.

7. Tables

Table 1: The 15 amino acids used in the 1:1 w:w protein:sucrose formulations along with the corresponding T_g' . DSC was used to determine the T_g' (from the mid-point on the thermogram) for 0.05:1 w:w amino acid:sucrose solutions.

Amino Acid (X)	0.05:1 X:SUC T_g' (°C)
Lysine	-37
Alanine	-36
Aspartic Acid	-35
Glutamic Acid	-35
Serine	-35
Arginine	-34
Glutamine	-34
Isoleucine	-34
Methionine	-34
Threonine	-34
Valine	-34
Citrulline	-33
Leucine	-33
Phenylalanine	-33
Histidine	-32

Table 2: Physical and chemical characteristics of the 15 amino acids used in the 1:1:0.3 protein:SUC:amino acid formulations.

Side Chain Classification	Amino Acid	Density (g/mL) ^a	Molar Volume (mol/mL)	pI ^b	Dipole Moment (D) ^b	Hydrophobicity ^c	Side Chain Flexibility ^c	Potential side chain H bonds ^c
Hydrophobic	Alanine	1.37*	65.0	6.1	0	0.806	Low	0
	Valine	1.27	92.3	6.0	0.06	0.923	Low	0
	Leucine	1.20	109.3	6.0	0.09	0.918	Moderate	0
	Isoleucine	1.17	112.1	6.0	0.07	1.000	Moderate	0
	Methionine	1.30*	114.8	5.7	1.80	0.811	High	0
	Phenylalanine	1.32	125.2	5.9	0.29	0.951	Moderate	0
Polar	Serine	1.55*	67.8	5.7	1.83	0.601	Low	3
	Threonine	1.46*	81.6	5.6	1.79	0.634	Low	3
	Glutamine	1.32	110.7	5.7	3.89	0.430	High	5
	Citrulline	1.29	135.8	5.9 ^e	-	-	-	-
Positive	Lysine	1.33*	109.9	9.5	9.07	0.263	High	3
	Histidine	1.41	110.1	7.6	4.04	0.548	Moderate	3
	Arginine	1.33	131.0	10.8	5.78	0.000	High	7
Negative	Aspartic Acid	1.64*	81.2	3.0	4.33	0.417	Moderate	4
	Glutamic Acid	1.57	93.7	3.1	6.13	0.458	High	4

a) Crystalline density values measured directly using He pycnometer (*) or obtained from literature values.

b) pI and dipole moment from (24). Citrulline pI from (28).

c) Hydrophobicity, side chain flexibility and potential side chain hydrogen bonds (29).

Table 3: Pearson's correlation coefficient results of the amino acid variables from Table 2.

Variables	Molar Volume	Side Chain Charge (Polarity)	Potential side chain H bonds	Hydrophobicity	pI	Dipole Moment
Molar Volume	-	-	-	-	-	-
Side Chain Charge (Polarity)	0.464	-	-	-	-	-
Potential side chain H bonds	0.111	0.147	-	-	-	-
Hydrophobicity	-0.030	-0.158	-0.911	-	-	-
pI	0.506	0.946	0.252	-0.198	-	-
Dipole Moment	0.216	0.249	0.738	-0.878	0.325	-
Side Chain Flexibility	0.696	0.140	0.432	-0.484	0.235	0.663

Values in bold are different from 0 with a significance level $\alpha=0.05$

Table 4: Multivariable analysis for ACT formulations.

Variable	Coefficient	Std. Error	p-value < 0.20
Intercept	0.123	0.285	0.675
Molar Volume	-0.324	0.168	0.083
Side Chain Charge (Polarity)	0.481	0.299	0.139

Table 5: Multivariable analysis for rHSA formulations.

Variable	Coefficient	Std. Error	p-value < 0.20
Intercept	0.029	0.461	0.951
Molar Volume	-0.612	0.319	0.081
Side Chain Charge (Polarity)	1.097	0.508	0.054

8. Figures

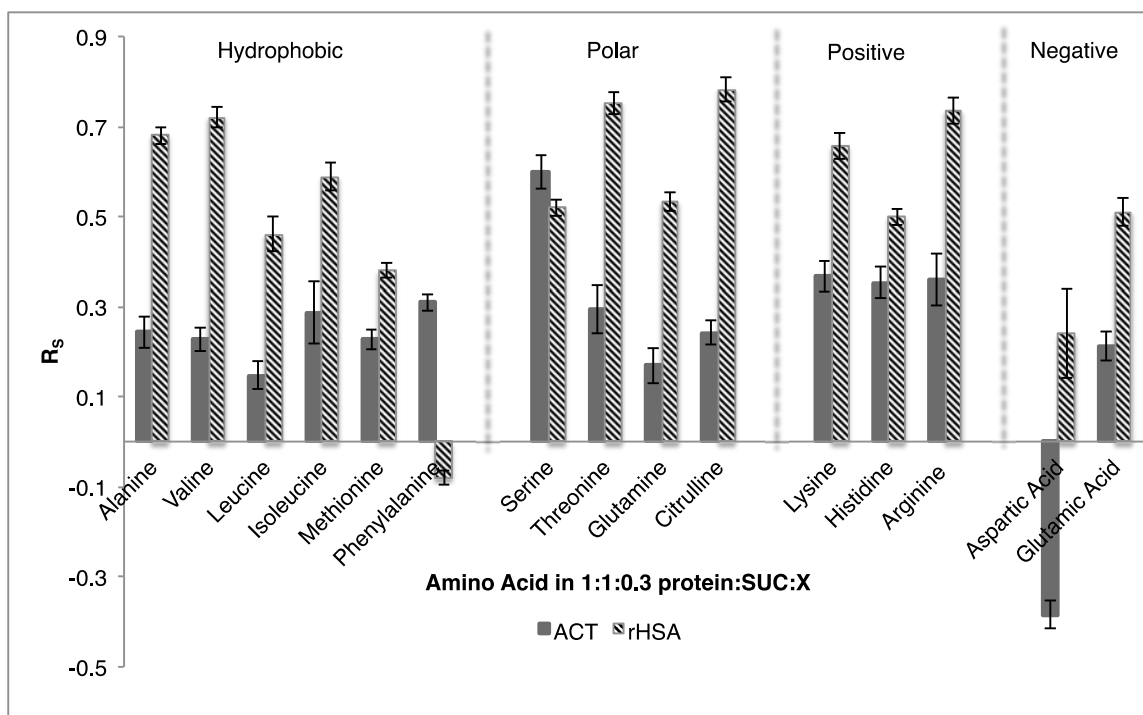


Figure 1: Stability results for the 1:1:0.3 ACT:SUC:X (gray bar) and rHSA:SUC:X (stripped bar) (X = amino acid) formulations after storage for two months at 50°C. The stabilization ratio (R_s) for each amino acid formulation is calculated from Equation 1. The formulations are grouped by amino acid side chain classification and in order of increasing molar volume. Error bars are standard errors of the mean.

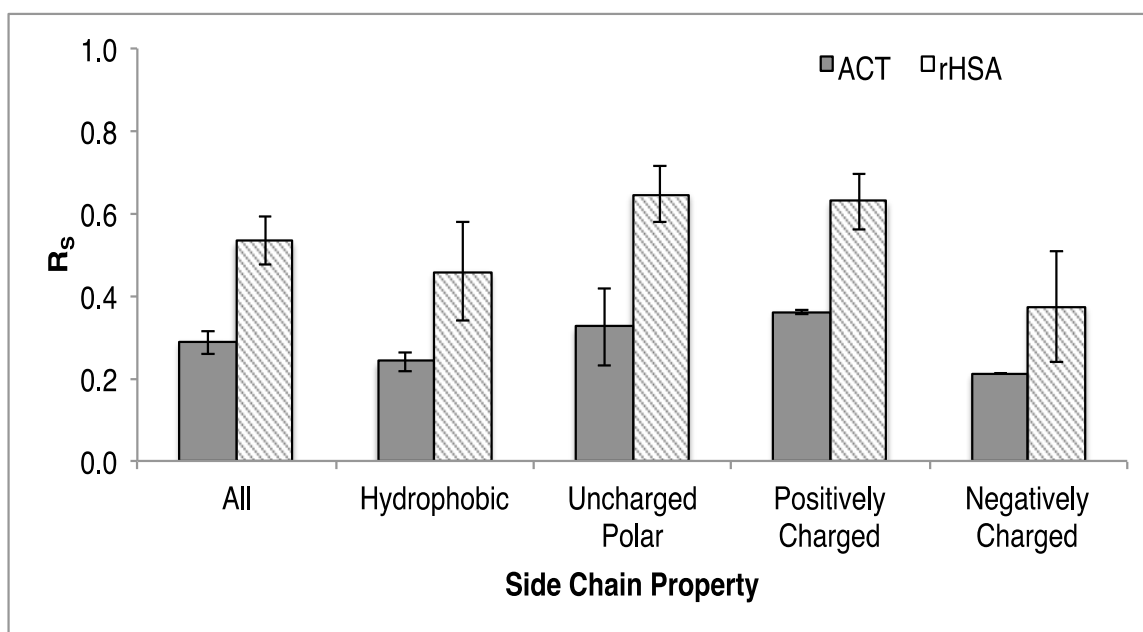


Figure 2: Comparison of side chain properties with the average stabilization ratio for each classification of amino acid. Error bars are standard errors of the mean. (Aspartic acid was not included in the ACT analysis due to the significantly high amount of aggregation.)

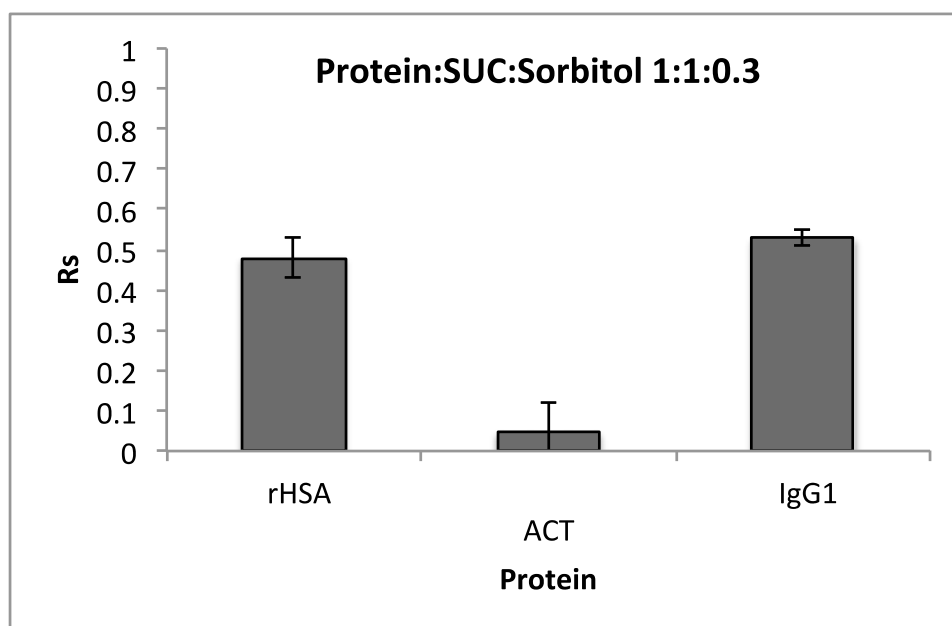


Figure 3: The percent stabilization of sorbitol for 1:1:0.3 protein:sucrose:sorbitol formulations. Where the proteins are: rHSA, ACT and IgG1. Error bars are standard errors of the mean. All data is for aggregation stability after storage at 50°C. IgG1 from (16).

Chapter 4

Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: II. Evaluation of Optimal Amino Acid Concentrations for Stabilizing rHSA:Sucrose Formulations

Abstract

The purpose of this study was to investigate the long-term storage stability of a sucrose-based protein formulation lyophilized with small molecule additives (i.e., amino acids, sorbitol) over a large concentration range. The optimal stabilizing amount of each amino acid was evaluated, and the potential correlation between the extent of stabilization and the additive chemical and physical properties was investigated. Formulations containing a protein (recombinant human serum albumin (rHSA)), sucrose (SUC) and 13 different additives (12 amino acids and sorbitol) were lyophilized at a weight ratio of 1:1:X Sucrose:rHSA:Additive ($X = 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8$). Protein aggregation after three months storage at 50°C was determined by size exclusion chromatography. The addition of additives with a low glass transition temperature (T_g), to a solid-state sucrose-based protein formulation is expected to result in plasticization and a decrease in the system T_g , which in turn would classically be expected to destabilize the product (due to an expected decrease in structural relaxation time). However, the addition of small molecules to the rHSA:SUC formulation resulted in significantly reduced aggregation compared to formulations with only sucrose. The concentration dependence of stabilization by the additive varied with the nature of the additive. Formulations with valine, leucine, glutamic acid, lysine, arginine and citrulline had improved stability at all concentrations. Alanine had improved stability for concentrations from 1:1:0.2 to 1:1:0.7 (rHSA:SUC:alanine), while aspartic acid had improved stability for concentrations above 1:1:0.2 (rHSA:SUC:aspartic acid). We find that the addition of any of the 12 amino acids or sorbitol to a model lyophilized sucrose-based protein

formulation reduced the amount of aggregation and significantly improved the storage stability, with some, but not all of the amino acids showing an optimum concentration for stabilization.

1. Introduction

Pharmaceutical proteins are often lyophilized to obtain acceptable storage stability. Excipients are necessary to adequately protect the protein from degradation during both process and storage(1), where sucrose is often used as the main stabilizer due to the ability to provide both thermodynamic and kinetic stabilization(2, 3). Recently, some research has focused on the further stabilizing of disaccharide-based protein formulations by the addition of a low level of “small molecule” additives, such as glycerol or sorbitol(4-6). Classically, the addition of these types of additives would be avoided in solid-state formulations due to the decrease in the system’s collapse temperature, making freeze-drying more difficult. The addition of small molecules is also expected to result in a decrease in the glass transition temperature (T_g) of the dried product and a subsequent decrease in the structural relaxation time, frequently considered predictive of decreased storage stability(7). It has been hypothesized that the addition of the small molecule additives resulted in improved stabilization as a result of the decreased matrix free volume and resulting suppression of motions on a small length scale and small time scale (i.e., suppression of fast dynamics)(6, 8). Besides polyols (e.g., glycerol and sorbitol) other small molecules have also been shown to stabilize solid-state protein formulations, including water and some amino acids(9-12).

In several studies involving small molecules added to protein formulations for freeze drying, stability improved over a range of concentrations, and in a few cases optimal stabilizing amounts could be identified. For example, the activity of two enzymes (ADH and YHRP) lyophilized in trehalose was investigated over a small range of glycerol

concentrations. It was found that the highest enzyme activity was obtained at 5 % w/w. Both systems without glycerol, and systems with 10 % w/w glycerol showed inferior storage stability(4). In a separate study, lyophilized disaccharide-based antibody formulations containing sorbitol showed improved storage stability with added sorbitol over the entire range of sorbitol studied (2 – 20 % of total solids, w/w) compared to formulations without sorbitol. No optimal stabilizing amount was found over the range of sorbitol studied(5). The same study also found improved physical and chemical stability of 1:1 w/w sucrose:IgG1 formulations at intermediate concentrations of water. Water contents between 0 and 5.2% were investigated, and optimal stability was found at 2.4% water(5). Similar results were found for another lyophilized sucrose-based monoclonal antibody formulation, where aggregation during storage was at a minimum at 3% residual water. The study did not find any improvement in stabilization against chemical degradation with intermediate residual moisture(9).

Studies presented in the first paper of this series demonstrated that sucrose-based protein formulations (1:1 w:w) freeze-dried with amino acid additives at a level of 13% (w/w) of total solids provide significant stabilization for nearly all amino acids investigated(13). This work showed that the extent of stabilization at this fixed concentration was somewhat dependent on several of the amino acid properties, including: molar volume, side chain charge (i.e., polarity), dipole moment, hydrophobicity and potential hydrogen binding sites on the side chain. However, the stabilization extent was somewhat protein specific. Since only one amino acid concentration was studied, the range of amino acid contents required to obtain optimal

stability or whether or not this range is sensitive to the nature of the amino acid was not addressed.

The objective of this study was to investigate the extent of stabilization for a range of amino acids concentrations added to a 1:1 (w:w) rHSA:Sucrose lyophilized formulation and to determine what concentration range of amino acid provided the highest stabilization for each amino acid. Several sorbitol concentrations in this model protein formulation were also studied for comparison to a previous literature report(5). The best stabilization ratio for each amino acid was compared to several physical and chemical properties of the amino acid molecule to establish if any properties related to the additives chemical and physical nature were critical for the stabilization.

2. Materials and Methods

2.1 Materials

Ultra-pure recombinant human serum albumin (rHSA) was purchased from Albumin Biosciences (Huntsville, AL). The material was recombinant protein with certified to have purity greater than 99% as determined by SDS-PAGE and IEF. The phosphate buffers, sucrose (SUC), sorbitol and all amino acids (Table 1) were purchased from Sigma (St. Louis, MO). All amino acids were of the neutral form and salt free. All excipients were of the highest grade available and used without further purification.

2.2 Sample Preparation

Samples were prepared as previously reported(13). Briefly, before freeze-drying, rHSA (MW = 66.4 kDa, pI = 4.7) was dialyzed against 2mM sodium phosphate buffer at pH 7 using a dialysis membrane with MWCO: 6-8,000 kDa (Spectrum Laboratories, Dominguez, CA). The final concentration of rHSA was 5 mg/mL, confirmed by UV absorbance at 280 nm. Solutions of 1:1:X (X =0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8) rHSA:sucrose:additive were prepared using bulk solutions of protein, sucrose, amino acid and/or sorbitol. The final pH of each solution was adjusted to pH 7 as needed. All solutions were filtered through 0.22 μ m filters and 1mL of the final solution was filled into 3 cc glass tubing vials and semi-stoppered with Daikyo Flurotech stoppers (West Pharmaceutical, Lionville, PA), which are low moisture release stoppers.

2.3 Lyophilization

Solutions were lyophilized in a Durastop Freeze dryer (FTS, SP Industries, Stone Ridge, New York). Using the Fox Equation and previously reported data(13), the T_g' values for the rHSA:SUC:additive formulations were estimated to be between -27 and -23°C. Thermocouples were placed in the bottom center of at least four vials per batch. The freeze-drying process is exactly the same as reported previously(13). All the formulations were freeze-dried as one batch to ensure essentially the same thermal history. After freeze-drying, samples were confirmed to be amorphous by polarizing light microscopy, as judged by no birefringence. Water content was between 0.1-0.3% (w/w) as determined by Karl-Fischer moisture titration (756 Coulometric Titrator, Metrohm, Riverview, FL).

2.4 Stability Studies

Long-term storage stability was evaluated by determining the total percent soluble protein aggregates after processing (time zero) and after three months storage at 50°C by size exclusion chromatography (SEC), as described previously(13). In general, two replicate samples were assayed both at time zero and at three months to check for reproducibility. The mean difference between replicate samples was 0.2%. Preliminary studies showed stability follows square root of time kinetics(7). Stability was assessed by determining the amount of measured total soluble aggregation during the storage stability study, ΔA . That is, the “initial” level of aggregate after freeze-drying (including in-process aggregation), was subtracted from the amount of measured total soluble aggregation after three months storage, to obtain the total percent aggregation during storage (ΔA) for each formulation. In order to evaluate the extent of stabilization, a stabilization ratio (R_s) for each formulation containing an additive (A^x) was calculated, as shown in Equation 1.

$$R_s = \frac{\Delta A^0 - \Delta A^x}{\Delta A^0} \quad (1)$$

Here, ΔA^0 is the increase in aggregation during storage for the base-line system without any additive and ΔA^x is the increase in aggregation during storage for the system with additive. A larger stabilization ratio (R_s) indicates better stabilization with the selected amino acid additive (A^x) compared to the sucrose-based protein formulation containing no additive (A^0). A value of unity means perfect stabilization, a value of zero means no

additional stabilization, and a negative value means the additive decreased the stabilization.

In order to rule out an amino acid stabilizing by acting as an antioxidant in the protein formulations, the percent total soluble aggregation was measured for 1:1:X (X = 0, 0.1, 0.4) rHSA:SUC:Serine formulations after storage at 50°C for three months with either ultra pure nitrogen or air (i.e., with oxygen, < 3% RH) in the vial headspace. Serine is not a suspected antioxidant, so if oxidation is an aggregation pathway for rHSA, the presence of oxygen in the headspace should lead to significantly greater aggregation with air in the headspace relative to the formulations with ultra pure nitrogen in the headspace.

2.5 Statistical Analysis

As previously reported(13), multivariable regression analysis was carried out to establish the correlation between the best stabilization ratio obtained by each amino acid and the independent amino acid properties using XLSTAT(Version 2013.3.01, Addinsoft, New York, NY). The variables used for analysis were molar volume, hydrophobicity and side chain charge (i.e., polarity). These variables provided an adequate representation of amino acid physical and chemical properties. Variables were considered significant at a p-value < 0.20 (80% confidence interval).

3. Results and Discussion

3.1 Stability Studies

After storage, all amino acid and sorbitol formulations were confirmed amorphous by polarizing light microscopy, as judged by no birefringence. For select formulations the water content was determined by Karl-Fischer moisture titration to be between 0.2-0.9% (w/w) after storage. The small increase in water content indicates that there was insignificant water transfer from the stopper to the cake over three months storage and therefore, it can be concluded that the aggregation during storage was not significantly influenced by variation in residual moisture.

The soluble % aggregation before freeze-drying was approximately 0.1%. After processing (time zero) the measured soluble aggregation (0.2%) was not significantly different (p -value < 0.05) for formulations both with and without the small molecule additives, indicating the amino acids and sorbitol did not alter stability during the freeze-drying process. The glass transition temperature (T_g) for all formulations was estimated from the Fox Equation(14) and temperature data from a previous study(13). All amino acid formulations at 1:1:0.8 have calculated T_g values much higher than the storage temperature of 50°C (Table 1). T_g values for formulations with amino acids at lower concentrations have equal or lower T_g values than those listed in Table 1. No visible shrinkage was observed after three months storage, indicating all formulations remained in the glassy state throughout the stability study.

The results of the three month stability study at 50°C for rHSA:SUC:additive formulations are shown in Figure 1, where the stabilization ratio for each amino acid additive is shown versus the ratio of amino acid added (i.e., the value of “X”) to the sucrose-based rHSA formulation. The amino acids studied were divided into four main groups based on the stability profile of each amino acid and the side chain properties of the amino acid in solution (Table 1). The classes are as follows: 1) No maximum stabilization reached, 2) Maximum stabilization reached and nonpolar side chain, 3) Maximum stabilization reached and polar side chain, and 4) Methionine. Sorbitol was grouped with the polar, side chain amino acids (group 3). The stability results for each group are discussed below:

1) No maximum stabilization reached (Figure 1a): This group consists of amino acids with charged side chains (arginine, lysine, aspartic acid, glutamic acid) and citrulline. Although citrulline is not charged, it is similar in structure to arginine. This and other potential grouping reasons are investigated in a later section. None of the amino acids in this group reach a maximum stabilization concentration, and stability increases monotonically with increasing level of amino acid over the entire range studied (0.1 to 0.8). For citrulline and the positively charged amino acids (lysine and arginine) the stability profiles are almost identical. The stability enhancement obtained is quite large; there is about a 5-fold improvement in stability compared with the formulation without any amino acid. The stability curves for the negatively charged amino acids, glutamic acid and aspartic acid, are not as stabilizing. At all concentrations studied, stability was improved, however; glutamic acid improved stability much better than aspartic acid, with

an almost 3-fold improvement in stability for glutamic acid formulations versus only a factor of 1.5 improvement in stability for aspartic acid formulations.

2) Maximum stabilization reached and nonpolar side chain (Figure 1b): This group consists of amino acids with nonpolar side chains that display a maximum stabilization amount. Formulations containing isoleucine, leucine and valine have improved stability over the entire concentration range studied ($X = 0.1 - 0.8$). Alanine has improved stability at all concentrations studied, except for 0.1; stability of the 1:1:0.1 rHSA:SUC:alanine formulation was not significantly different ($p\text{-value} < 0.05$) from the formulation with only sucrose. Isoleucine has an optimal stabilization range from 0.2-0.4. Alanine has a large optimal stability range, from 0.2-0.7. Leucine appears to stabilize in the ranges of 0.1-0.3 and 0.7-0.8 while valine stabilizes in concentration range 0.4-0.7. The magnitude of stabilization for all the non-polar molecules averages about a factor of 2.5, relative to the formulation without any amino acid.

3) Maximum stabilization reached and polar side chain (Figure 1c): The amino acids with polar side chains and sorbitol formulations all reach a maximum stabilizing amount. At higher concentrations of threonine, there is more than a 5-fold improvement in stability. This is similar to citrulline (Figure 1a); however, the increase in stability is not linear. For the serine formulations, there appears to be an optimal stabilizing range between 0.3-0.6, with stabilization two times greater than the sucrose only formulation. Previously, sorbitol was shown to improve the stability of a disaccharide-based protein formulation; however, the concentration range studied did not exceed 13% and no

optimal stabilizing range was observed(5). For this study, the sorbitol formulations show an optimal stabilizing amount of about a factor of two for concentrations of 0.3-0.6, similar to the serine formulations.

4) Methionine: Methionine has improved stability over the small optimal range of 0.1-0.3, while higher amounts of methionine destabilized the formulation. Methionine is grouped separately from the noncharged amino acids due to the large difference in the stabilization profile. The potential antioxidation effects of methionine are discussed in section 4.3.

All additives studied (amino acids and sorbitol) improved the aggregation stability of a lyophilized sucrose-based rHSA formulation. Table 1 summarizes the range of stabilizing amounts for each additive. It is clear that stabilization occurs over a wide range of additive concentrations. For those systems not showing a maximum in stabilization ratio, an optimal stabilizing amount may be present at higher concentrations of additive beyond the concentration range investigated. However, at higher additive concentrations there is also an increased probability of crystallization for certain additives (i.e., threonine, citrulline, aspartic acid, glutamic acid, lysine, arginine), as well as having a very low T_g .

3.2 Maximum Stabilization

The maximum amount of stabilization was determined for each amino acid as the highest stabilization ratio (R_s) observed for each additive. The average improvement in

stability for all amino acids studied was close to 60%, meaning a factor of 2.5 less aggregation compared to the formulation with only sucrose and rHSA (Figure 2). The positively charged amino acids provide the greatest extent of stabilization, with a maximum stabilization at 80%, Figure 2. The effect of arginine on protein stability has been increasingly studied in literature, particularly in the solution state(15-18). One solid-state spectroscopic study of arginine freeze-dried with a monoclonal antibody suggested stabilization of the protein was a result of arginine directly interacting with the protein surface(19). It was hypothesized that ion-dipole interactions, electrostatic interactions and hydrogen bonding occurred between the protein and the amino acid, likely largely a result of the charged state of arginine. It is possible that lysine could also stabilize in the same manner as arginine. As shown in Figure 1a, arginine and lysine have nearly identical curve shapes, suggesting similar stabilization by the positively charged molecules.

Charge may not be the only significant factor in stabilization. Citrulline also has a curve shape very similar to arginine and lysine (Figures 1a) but carries no net charge at the pH studied. The structures of arginine and citrulline only differ by a carbonyl and a guanidine functional group, but at the pH studied (in solution), arginine is charged while citrulline has a neutral side chain. The overlap of the stability curves for citrulline, arginine and lysine suggests that the chemical structure of the additive, independent of net charge, may also be important to the mechanism of stabilization. From our previous study, it was concluded that both structure and side chain charge (i.e., polarity) are important factors to consider(13).

3.3 Methionine as an Antioxidant

Methionine is an amino acid commonly used in liquid pharmaceutical formulations for its antioxidant properties(20, 21). To determine if the aggregation of rHSA in the presence of methionine is due to prevention of oxidation by methionine, the stability of 1:1:X (X = 0, 0.1, 0.4) rHSA:SUC:serine formulations were evaluated in the presence of oxygen and compared to formulations studied under nitrogen. We emphasize that serine is not a suspected antioxidant. The aggregation behavior is essentially the same in the presence and absence of oxygen (Figure 3). This observation indicates that oxidation is not a significant degradation pathway for producing aggregation in rHSA, and therefore, the stabilizing mechanism for methionine (or any of the other amino acids studied) is not related to its antioxidant properties.

3.4 Multivariable Regression Analysis

A multiple regression analysis was conducted to determine the effect of side chain charge (i.e., polarity), molar volume and hydrophobicity on the maximum amount of stabilization and to determine if a reasonable prediction of stability could be obtained from these amino acid properties via the empirical model. The variables were investigated in previous work(13), and it was determined that lower molar volume and positively charged amino acids contribute to stability at the ratio of 1:1:0.3 rHSA:SUC:amino acid. For the present analysis, the maximum amount of stabilization for each amino acid was used as the dependent variable in the multivariable analysis. When all amino acids were run in the analysis, the results did not reveal any significant contributions from any of the independent variables (results now shown). For this

reason, the multivariable analysis was run for the grouped amino acids (described above).

The multivariable analysis results for amino acids with charged side chains and citrulline (group 1) are shown in Table 2a. Interactions were investigated, however, were not significant. Molar volume is considered highly predictive of the stability. Interestingly, the coefficient indicates that a larger molar volume leads to a higher stabilization ratio. This is similar to the conclusions made in the previous research, where larger amino acids stabilized to a greater extent(13). It just happens that the larger amino acids are also positively charged. In the case of citrulline, which is not charged, the stability is purely attributed to the size. The average experimental R_s and average model R_s were both 0.722. The standard deviation of the mean for the experimental R_s values was ± 0.218 while the standard deviation of the mean for the calculated R_s values was ± 0.199 . The average relative deviation of the calculated R_s from the experimental R_s was 0.027. This indicates that the accuracy of the model is similar to the accuracy of the experimental data, and therefore, the significance of molar volume is real for the lysine, arginine, citrulline, glutamic acid and aspartic acid.

The multivariable analysis for the amino acids with nonpolar side chains (group 2) is shown in Table 2b. Both hydrophilicity and molar volume contribute significantly to the stabilization ratio and interactions were found not to be significant. A smaller molar volume and more hydrophobic additive improves the stability the best. This makes sense given the nature of the group of amino acids in this analysis. When methionine

was included in the analysis the variables became insignificant, indicating the behavior of methionine is an outlier compared to the other additives. The average experimental R_s and average model R_s were 0.615 and 0.613, respectively. The standard deviation of the mean for the experimental R_s values was ± 0.0057 while the standard deviation of the mean for the calculated R_s values was also ± 0.0057 . The average relative deviation of the calculated R_s from the experimental R_s was -0.002. This indicates that the accuracy of the model is the same as the accuracy of the data and this model is a very good predictor of stability and the significance of molar volume and hydrophobicity is real for amino acids with nonpolar side chains.

4. Conclusions

This study demonstrated that the stabilizing effect of various amino acids and sorbitol on the storage stability of freeze-dried sucrose-based protein formulations is not necessarily restricted to a narrow range of additive concentrations. Stability of rHSA in 1:1 w/w formulations with sucrose is enhanced by addition of amino acids and sorbitol over a large range of additive compositions, with no clear optimal amount of additive for most of the amino acids studied. Maximum stability enhancement can be very large; for several amino acids (threonine, citrulline, arginine, lysine) there was about a five-fold improvement in stability. It seems that the extent of stabilization is dependent on the molar volume, side chain charge, and hydrophobicity. It is apparent that the stabilizing range is very sensitive to the additive used in the formulation.

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6. References

1. Jorgensen L, Hostrup S, Moeller EH, Grohgan H. Recent Trends in Stabilising Peptides and Proteins in Pharmaceutical Formulation - Considerations in the Choice of Excipients. *Expert Opinion*. 2009;6(11):1219-30.
2. Wang B, Tchessalov S, Cicerone MT, Warne NW, Pikal MJ. Impact of Sucrose Level on Storage Stability of Proteins in Freeze-Dried Solids: II. Correlation of Aggregation Rate with Protein Structure and Molecular Mobility. *Journal of Pharmaceutical Sciences*. 2008;98(9):1-22.
3. Wang B, Tchessalov S, Warne NW, Pikal MJ. Impact of Sucrose Level on Storage Stability of Proteins in Freeze-Dried Solids: I. Correlation of Protein-Sugar Interaction with Native Structure Preservation. *J Pharm Sci*. 2009;98:3131-44.
4. Cicerone MT, Tellington A, Trost L, Sokolov A. Substantially Improved Stability of Biological Agents in Dried Form: The Role of Glassy Dynamics in Preservation of Biopharmaceuticals. *BioProcess International*. 2003;V1(1):36-47.
5. Chang L, Shepherd D, Sun J, Tang X, Pikal MJ. Effect of Sorbitol and Residual Moisture on the Stability of Lyophilized Antibodies: Implications for the Mechanism of Protein Stabilization in the Solid State. *Journal of Pharmaceutical Sciences*. 2005;94(7):1445-55.
6. Chieng N, Cicerone MT, Zhong Q, Liu M, Pikal MJ. Characterization of Dynamics in Complex Lyophilized Formulations: II. Analysis of Density Variations in Terms of Glass Dynamics and Comparisons with Global Mobility, Fast Dynamics, and Positron Annihilation Lifetime Spectroscopy (PALS). 2013.
7. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang X, et al. Mechanism of Protein Stabilization by Sugars During Freeze-Drying and Storage: Native Structure

Preservation, Specific Interaction, and/or Immobilized in a Glassy Matrix? Journal of Pharmaceutical Sciences. 2005;94(7):1427-44.

8. Cicerone MT, Soles CL. Fast Dynamics and Stabilization of Proteins: Binary Glasses of Trehalose and Glycerol. Biophysical Journal. 2004;86:3836-45.

9. Breen ED, Curley JG, Overcashier DE, Hsu CC, Shire SJ. Effect of Moisture on the Stability of a Lyophilized Humanized Monoclonal ANTibody Formulation. Pharm Res. 2001;18(9):1345-53.

10. Al-Hussein A, Gieseler H. Investigation of Histidine Stabilizing Effects on LDH During Freeze-Drying. J Pharm Sci. 2012;102(3):813-26.

11. Paik SH, Kim YJ, Han SK, Kim JM, Huh JW, Park YI. Mixture of Three Amino Acids as Stabilizers Replacing Albumin in Lyophilization of New Third Generation Recombinant Factor VIII GreenGene F. Biotechnol Prog. 2012;28(6):1517-25.

12. Lale SV, Goyal M, Bansal AK. Development of Lyophilization Cycle and Effect of Excipients on the Stability of Catalase During Lyophilization. Int J Pharm Investig. 2011;1(4):214-21.

13. Forney-Stevens KM, Bogner RH, Pikal MJ. Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: Screening of 15 Amino Acids in Two Model Protein Formulations. 2013.

14. Donth E-J. Relaxation and Thermodynamics in Polymers: Glass Transition. Berlin: Akademie Verlag; 1992. 355 p.

15. Nakakido M, Kudou M, Arakawa T, Tsumoto K. To be Excluded or to Bind, that is the Question: Arginine Effects on Proteins. Curr Pharm Biotechnol. 2009;10(4):415-420.

16. Lange C, Rudolph R. Suppression of Protein Aggregation by L-Arginine. *Curr Pharm Biotechnol*. 2009;10:408-14.
17. Baynes BM, Wang DIC, Trout BL. Role of Arginine in the Stabilization of Proteins Against Aggregation. *Biochemistry*. 2005;44:4919-25.
18. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T. Role of Arginine in Protein Refolding, Solubilizing, and Purification. *Biotechnol Prog*. 2004;20:1301-8.
19. Tian F, Middaugh CR, Offerdahl T, Munson E, Sane S, Rytting JH. Spectroscopic Evaluation of the Stabilization of Humanized Monoclonal Antibodies in Amino Acid Formulations. *Int J Pharm*. 2007;335:20-31.
20. Lam XM, Yang JY, Cleland JL. Antioxidants for Prevention of Methionine Oxidation in Recombinant Monoclonal Antibody HER2. *J Pharm Sci*. 1997;86:1250-5.
21. Wade AM, Tucker HN. Antioxidant Characteristics of L-Histidine. *J Nutritional Biochem*. 1998;9(6):308-15.

7. Tables

Table 1: The maximum stabilizing concentration for the 1:1:X rHSA:SUC:additive formulations along with the molar volume, hydrophobicity, and side chain property for each amino acid studied.

Stability Profile	Additive	Side Chain Property	Molar Volume*	Hydrophobicity**	Maximum Stabilization Concentration
No Maximum Stabilization	Aspartic Acid	Negatively Charged	81.2	0.417	> 0.8
	Glutamic Acid		93.7	0.458	> 0.8
	Lysine	Positively Charged	109.9	0.263	> 0.8
	Arginine		131.0	0.000	> 0.8
	Citrulline	Polar Uncharged	135.8	-	> 0.8
Maximum Stabilization Reached, Non Polar Side Chain	Alanine	Hydrophobic	65.0	0.806	0.6
	Valine		92.3	0.923	0.6
	Leucine		109.3	0.918	0.2
	Isoleucine		112.1	1.000	0.3
Maximum Stabilization Reached, Polar	Serine	Polar Uncharged	67.8	0.601	0.4
	Threonine	-	81.6	0.634	0.6
	Sorbitol		182.2	-	0.3
Methionine	Methionine	Hydrophobic	114.8	0.811	0.1

* Molar volume from (13)

**Hydrophobicity (14)

Table 2: a) Multivariable analysis for amino acids with no maximum stabilization (group 1, Table 1) reached and **b)** Multivariable analysis for amino acids with nonpolar side chain (group 2, Table 2) and maximum stabilization reached.

a)

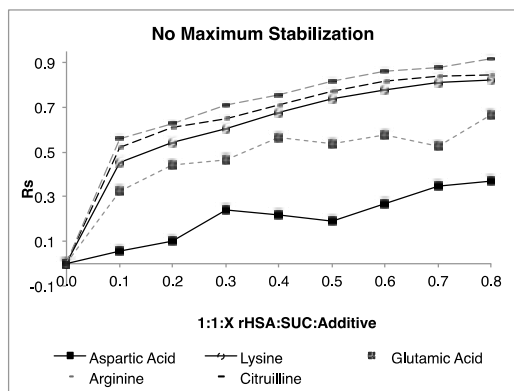
Variable	Coefficient	Std. Error	p-value
Intercept	-0.264	0.261	0.385
Molar Volume	0.922	0.240	0.031

b)

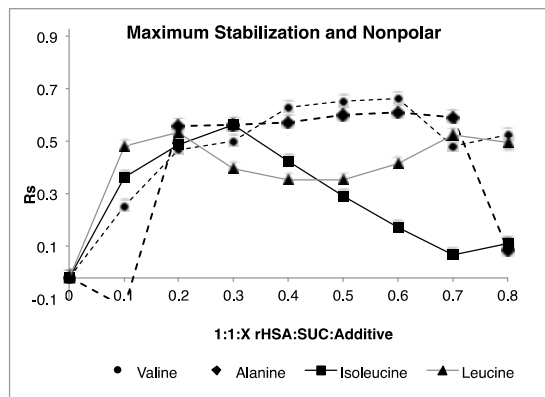
Variables	Coefficient	Std. Error	p-value
Intercept	0.457	0.057	0.078
Molar Volume	-0.609	0.076	0.079
Hydrophobicity	0.399	0.060	0.095

8. Figures

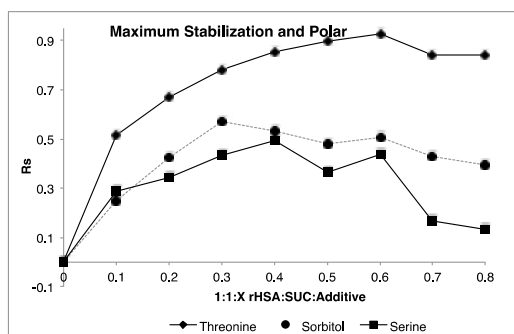
a)



b)



c)



d)

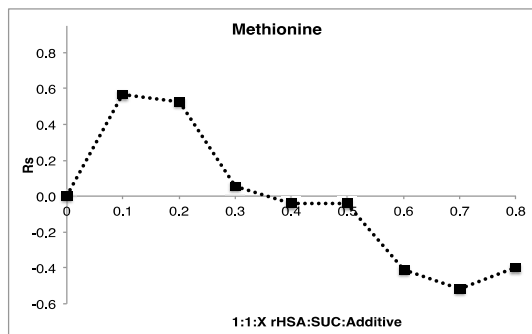


Figure 1: Three month storage stability study at 50°C for 1:1:X rHSA:SUC:Additive. The stabilization ratio (R_s) is calculated from Equation 1. Error bars are the standard error of the mean and the same size as the symbols. Results are grouped by similar stability profiles and amino acid side chain properties: a) No maximum stabilization observed, b) maximum stabilization reached and non-polar side chains, c) maximum stabilization reached and polar side chains, d) methionine. Sorbitol is grouped with the polar side chains.

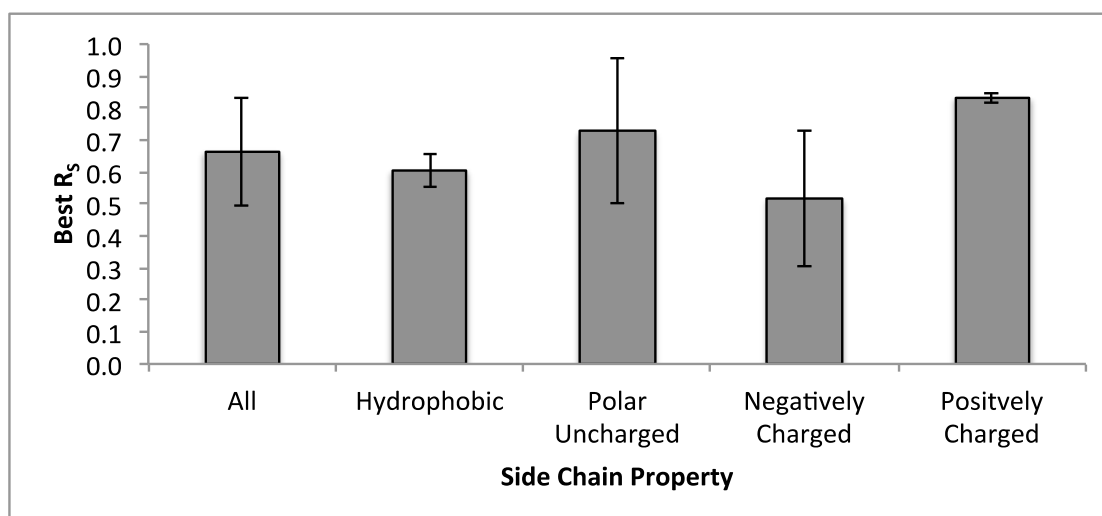


Figure 2: Comparing the best stabilization ratio (R_s) for each amino acid group. Sorbitol is grouped with the polar uncharged amino acids. The error bars are for the standard error of the means.

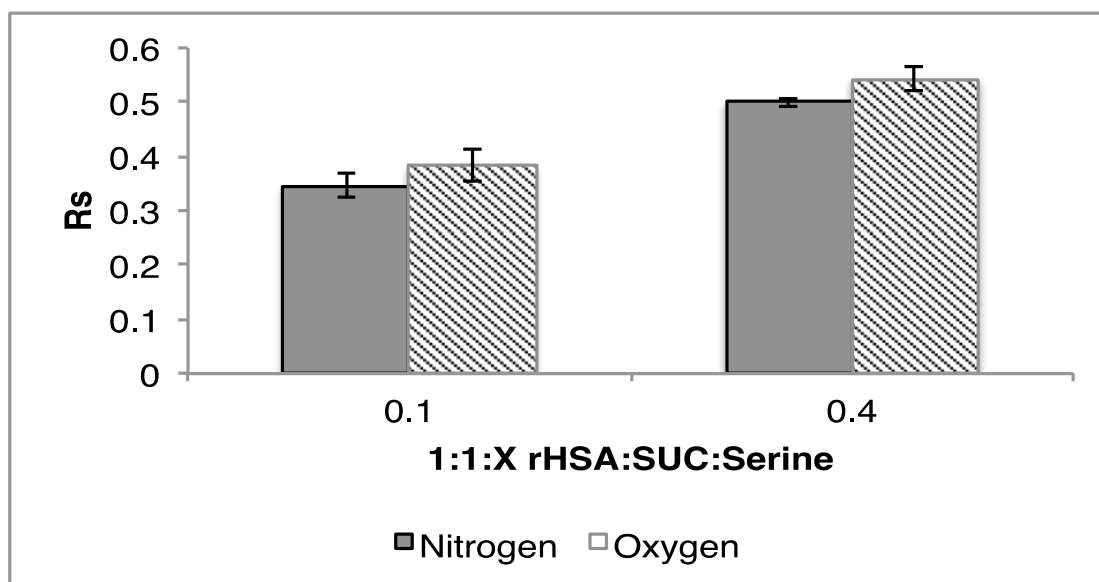


Figure 3: Test to determine if the instability of rHSA is a result of oxidation. Aggregation was measured after three month storage at 50°C for 1:1:X rHSA:SUC:serine (X 0.1, 0.4) formulations. Vials contained either nitrogen or oxygen in the headspace. The stabilization ratio was determined from Equation 1. The error bars are the standard error of the mean.

Chapter 5

Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: III. Correlations of rHSA Stability in rHSA:Sucrose:Amino Acid Systems with Selected Measures of Mobility

Abstract

The purpose of this research was to investigate the effect of mobility and free volume in sucrose-based protein formulations lyophilized with amino acids and to apply these results to better understand the impact of “small molecule” additives on long-term storage stability. Formulations containing sucrose, recombinant human serum albumin (rHSA) and an amino acid (i.e., serine, methionine) were lyophilized at a ratio of 1:1:X ($X = 0, 0.1, 0.4, 0.8$) rHSA:SUC:amino acid. Soluble aggregation was measured by SE-HPLC after two and three months storage at 50°C and rate constants were shown to follow square root of time kinetics. Modulated DSC was used to determine the glass transition temperature (T_g) for each formulation, structural relaxation time was studied by Thermal Activity Monitor (TAM), and the fast dynamics of each system was characterized by neutron backscattering. Variations in free volume and apparent specific volumes were estimated using densities measured by a gas pycnometer. Free volume hole size was measured directly by Positron Annihilation Lifetime Spectroscopy (PALS). Optimal stability was found to occur at 1:1:0.1 for the methionine series and at 1:1:0.4 for the serine series, with improved stability of about a factor of two compared to formulations without any amino acid additive. Both the T_g and the structural relaxation time decreased with increasing amounts of serine, and there was no significant change in global motions for the methionine formulations, indicating no correlation between stability and global motions. For the serine series, the slowest motions on a nanosecond timescale did correspond to the formulation with the best stability, 1:1:0.4, but the overall correlation between stability and fast dynamics was poor. For the methionine

series, there was essentially no correlation between stability and fast dynamics. Neither results from the density analysis nor the PALS hole sizes show any obvious correlation with stability. While the stability of lyophilized sucrose-based rHSA was vastly improved by the addition of amino acids, there was no consistent correlation between the various measures of mobility or “free volume” and stability. In general, amino acids can be used to further improve the long-term stability of lyophilized protein formulations, but it was not possible to assign a single cause for this effect.

1. Introduction

Lyophilized protein formulations often include stabilizing excipients, such as sucrose, to achieve long-term storage stability(1). The stabilizers protect the protein during both process and storage, by minimizing chemical and physical degradation. There are two main hypotheses that have been proposed to explain the mechanism of stabilization by the excipients in the formulation. The “water substitution” concept is based on the excipient forming hydrogen bonds with specific areas of the protein surface, taking the place of water, and providing conformational stabilization, where unfolding of the protein is thermodynamically inhibited. Alternately, the “glass dynamics” hypothesis is based on the stabilizer forming a rigid matrix in which the protein is molecularly dispersed, thereby kinetically inhibiting reactions from occurring regardless of the thermodynamics of protein structural stability(2). While both mechanisms of stabilization have been well studied in the literature, the focus of this research is on the role of glass dynamics and molecular mobility in the protein formulation and the potential relationship to storage stability.

Often the glass transition temperature (T_g) is considered to be a key indicator of stabilization for a protein formulation. However, there are many examples where T_g is not a good indicator of stability and an excipient with a high T_g (e.g., HES, dextran), while considered a good glass former, may not provide the needed stabilization(3-5). It has been suggested that structural relaxation time, measured by isothermal calorimetry, can provide the best measure of global dynamics(6-8), though the correlations of structural relaxation time with storage stability are often weak(9). Recently, it has been proposed

that the long-term stability of amorphous pharmaceuticals is controlled by dynamics on a much faster timescale than structural relaxation(10-13). Studies involving the addition of small molecule additives (e.g., sorbitol, glycerol) have shown the decrease in dynamics on a nanosecond timescale, indicative of suppressed local motions within the glassy solid. The addition of the same low molecular weight additives also improved the storage stability of the protein formulations, even though enthalpy relaxation times and T_g were decreased (indicating an increase in global mobility)(8, 12).

The mechanism of stabilization by the “small molecule” additives may be rationalized in terms of classical free volume theory. In an amorphous matrix, there is a significant amount of free volume available for small movements of molecules, compared to the crystalline state, which by definition has no free volume. Movement of a single atom or functional group is related to the amount of free volume available nearby. With less free volume, motions require concerted movement of neighboring molecules (i.e., cooperativity in motion). Due to the small activation energy needed, local motions can readily occur within the glass and at storage well below the T_g , and it is these motions that have been linked to chemical and physical degradation(14). It is proposed that the small molecules (e.g., sorbitol and glycerol) can fit into the open spaces in the amorphous matrix, thereby decreasing the free volume and suppressing the local motions. The decrease in the local motions has been correlated to increased storage stability(8).

The stabilization of model lyophilized protein formulations with the addition of small molecule additives has been previously studied in this laboratory (15-17). It was found that the addition of sorbitol and amino acids, at several concentrations, to sucrose-based protein formulations greatly improved the long-term storage stability. If the amino acids stabilize in a behavior similar to the polyol formulations, the mechanism of stabilization may be a result of a decrease in free volume and a subsequent suppression of mobility on a nanosecond timescale.

The purpose of this study was to investigate various measures of mobility (enthalpy relaxation time and mean amplitudes of motion from neutron scattering) and free volume (change in volume on mixing and free volume hole size by PALS) for selected rHSA:sucrose:amino acid formulations and rHSA:sucrose:sorbitol formulations. The measures of mobility (i.e., dynamics, free volume) were compared to the aggregative storage stability for rHSA:sucrose:additive formulations in order to investigate the mechanism of stabilization by the addition of the amino acids methionine and serine.

2. Materials and Methods

2.1 Materials

Ultra-pure recombinant human serum albumin (rHSA) was purchased from Albumin Biosciences (Huntsville, AL). The material was recombinant protein certified to have purity greater than 99% as determined by SDS-PAGE and IEF. The phosphate buffers, sucrose (SUC), sorbitol, serine and methionine were purchased from Sigma (St. Louis,

MO). All excipients were of the highest grade available and used without further purification. Methionine and serine were of the neutral form and salt free.

2.2 Sample Preparation

Samples were prepared using the procedure previously described(15). Briefly, before freeze-drying, rHSA (MW = 66.4 kDa, pI = 4.7) was dialyzed against 2mM sodium phosphate buffer at pH 7 using a dialysis membrane with MWCO: 6-8,000 kDa (Spectrum Laboratories, Dominguez, CA). After dialysis, the protein concentration was confirmed to be 5 mg/mL by UV absorbance at 280 nm (Cary 50 Bio, Varian). Separately, bulk solutions of sucrose, sorbitol, methionine and serine were prepared in 2mM phosphate buffer at pH 7. Solutions of 1:1:X (X = 0, 0.1, 0.4, 0.8) for rHSA:sucrose:serine, 1:1:X (X = 0, 0.1, 0.4) for rHSA:sucrose:methionine and 1:1:X (X = 0, 0.1, 0.3, 0.5) for rHSA:sucrose:sorbitol were prepared. Before freeze-drying, all solutions were filtered through 0.22 μ m filters, and 5mL of the final solution was filled into 20 cc glass tubing vials and semi-stoppered with Daikyo Flurotech stoppers (West Pharmaceutical, Lionville, PA), which are low moisture release stoppers.

2.3 Lyophilization

Solutions were lyophilized in a Durastop Freeze dryer (FTS, SP Industries, Stone Ridge, New York) as described previously(15). All the formulations were freeze-dried as one batch to ensure the same thermal history. After processing, the formulations showed no visible signs of collapse and were confirmed to be amorphous by polarizing light microscopy, as evidence by no birefringence. Water content was between 0.1-0.3%

(w/w) as determined by Karl-Fischer moisture titration (756 Coulometric Titrator, Metrohm, Riverview, FL).

2.4 Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (Q1000) from TA Instruments (New Castle, DE) was used to determine the glass transition temperature (T_g) and the change in heat capacity (ΔC_p) at T_g . The same method was used as described previously(15). Briefly, T_g and ΔC_p were determined by modulated DSC using a ramp rate of 1°C/min, amplitude of +/- 0.82°C and frequency of 80 seconds. All samples were prepared at low humidity conditions (< 3 %RH). The T_g values were taken as the midpoint of the transition on the DSC thermogram.

2.5 Density Measurements by Gas Pycnometer

Densities of crystalline serine, crystalline methionine, freeze-dried rHSA:SUC:Serine (1:1:X, X = 0, 0.1, 0.4, 0.8) and freeze-dried rHSA:SUC:Methionine (1:1:X, X = 0, 0.1, 0.4) formulations were measured using an AccuPyc 1330 helium pycnometer (Micrometrics, Norcross, GA), which has been described previously(18). All procedures were carried out in a glove bag with controlled humidity (< 3% RH). Briefly, 100 mg of sample was gently packed into a dry 1 mL aluminum cup, and the cup was transferred to the pycnometer for measurement. All measurements were carried out at room temperature, 23°C. Each sample was measured 20 times, with 99 purges and the obtained density value was the average of the measurements. Three separate

measurements were performed for each formulation, and the standard error of the mean was about 0.2%.

2.6 Calculation of Volume Change on Mixing

A volume change on mixing was calculated to determine if there was a loss of free volume upon addition of the amino acids to the rHSA:SUC formulation. The description of this type of analysis has been described in detail(8). Briefly, the densities of the pure components (either in the amorphous or crystalline state) were used to calculate the initial specific volume (v'_0), shown in Equation 1,

$$v'_0 = \sum_i w_i \cdot v'_i = \sum_i w_i / d_i \quad (1)$$

where w_i is the weight fraction of component i of specific volume, v'_i , and d_i is the density of component i . For the mixtures, the specific volume is $v' = 1/d_m$, where d_m is the density of the mixture. The volume change on mixing one gram of total material ($\Delta v'_m$) is determined from Equation 2.

$$\Delta v'_m = v' - v'_0 \quad (2)$$

If there is a loss in free volume on mixing, $\Delta v'_m$ will be less than zero.

2.7 Calculation of Apparent Specific Volume

Apparent specific volume was calculated as another means of assessing the volume change on adding the “small molecule” solute to the solvent consisting of the

rHSA:sucrose system. This analysis is described in detail in a previous report(8).

Apparent specific volume (Φ'_V) is defined as:

$$\Phi'_V = \frac{1/d - w_1 \cdot v_1'^0}{w_2} \quad (3)$$

In Equation 3, d is the density of the mixture (1:1:X rHSA:SUC:amino acid), w_1 is the weight fraction of component 1 (i.e., 1:1 rHSA:SUC), w_2 is the weight fraction of component 2 (i.e., the amino acid) and $v_1'^0$ is the specific volume of component 1. The partial specific volume, \bar{V}_2' , may be calculated from the apparent specific volume and the concentration dependence of the apparent specific volume, Equations 4, 5, and 6.

$$\bar{V}_2' = w_2 \cdot \frac{\partial \Phi'_V}{\partial w_2} + \Phi'_V \quad (4)$$

$$\Phi'_V = \text{intercept} + \text{slope} \cdot \ln(w_2) \quad (5)$$

$$\bar{V}_2' = \text{slope} + \Phi'_V \quad (6)$$

Note that if the apparent specific volume is essentially independent of composition (w_2), the slope is zero and the apparent specific volume and partial specific volume are essentially the same. Such was the case with the systems studied in this research. The apparent specific volume can be compared with the specific volume of the crystalline solute. Observing an apparent specific volume less than the specific volume of the crystalline solute would indicate a reduction in free volume of the solvent upon addition of solute (i.e., the amino acid).

2.8 Stability Studies

Long-term storage stability was evaluated by determining the percent total soluble protein aggregates after processing (time zero) and after two and three months storage at 50°C. As previously described(15), the percentage of soluble aggregate was determined by size exclusion chromatography (SEC). After reconstitution, all vials were visually clear. In general, two replicate samples were assayed at each time point (0, 2, 3 months) to check for reproducibility. The mean difference between replicate samples was 0.2%.

As is common with amorphous protein formulations, aggregation followed stretched exponential kinetics(1, 17). The aggregation rate constants were obtained by fitting stability data to “square root of time” kinetics (Equation 7).

$$\%P = P_o + k\sqrt{t} \quad (7)$$

P_o is the initial level of degradation product(s) and k is the effective rate constant, which is determined from the percentage of soluble aggregation (%P) versus the square root of time (\sqrt{months}). Figure 1 shows an example of the fitting results for 1:1 rHSA:SUC formulations ($R^2 = 0.9999$ and slope standard error is 0.013). The average standard error in the slope for all formulations was 0.027%.

For sorbitol, only one storage time (3 months at 50°C) was used to assess degradation during storage. Here, as was done previously(15, 16), the amount of measured total soluble aggregation after freeze-drying (in-process aggregation) was subtracted from

the amount of measured total soluble aggregation after three months storage, to obtain the total percent aggregation during storage (ΔA) for each formulation. In order to evaluate the extent of stabilization with an additive (ΔA^x), compared to the base protein-sucrose formulation (ΔA^0), a stabilization ratio (R_s) for each formulation, as shown in Equation 8.

$$R_s = \frac{\Delta A^0 - \Delta A^x}{\Delta A^0} \quad (8)$$

Here, ΔA^0 is the increase in aggregation during storage for the base-sucrose protein system without any additive and ΔA^x is the increase in aggregation during storage for the system with additive. A stabilization ratio (R_s) of unity indicates no stabilization by addition of the “small molecule”, and $R_s > 1$ indicates better stabilization with the selected amino acid additive (A^x) compared to the sucrose-based protein formulation containing no additive (A^0).

All formulations were confirmed amorphous after three-month storage by polarized light microscopy. For select formulations the water content was determined by Karl-Fischer moisture titration to be between 0.2-0.9% (w/w) after storage, indicating there was insignificant water transfer from the stopper to the cake over three months storage.

2.9 Neutron Scattering Characterization of Fast Dynamics

To characterize the fast dynamics in the freeze-dried protein formulations, we used the high flux backscattering (HFBS) spectrometer at the National Institute of Standards and

Technology (NIST) Center for Neutron Research (CNR). The spectrometer was operated in the fixed-window scanning mode, where elastic scattering was recorded as a function of Q over a temperature range of 4 – 330 K. The sample was heated at 1 K/min and all temperatures were below the glass transition temperature of the formulations studied. For each sample, 300 mg was loaded and sealed into samples cells in a He purged vacuum glove box. The analysis of the elastic scattering intensity was preformed using DAVE software(19) and as described previously(8). The Q -dependence of the incoherent elastic scattering intensity was analyzed to give the hydrogen-weighted Debye-Waller factors and $\langle u^2 \rangle$ values. For final analysis, the hydrogen-weight mean-square atomic displacement $\langle u^2 \rangle$ was obtained over the temperature range of 4-330K. Specifically, for this study, the interest in $\langle u^2 \rangle$ was mainly at the temperature used for assessing storage stability, 50°C. Errors in $\langle u^2 \rangle$ were calculated form the standard devastation of the fitted (smoothed) curves.

2.10 Positron Annihilation Lifetime Spectroscopy

Positron Annihilation Lifetime Spectroscopy (PALS) was used to directly measure the free volume of the freeze-dried rHSA:SUC:serine formulations. The measurements were carried out at the positron facility of the North Carolina State University with a typical fast-fast bulk PALS system, as described previously(8). Briefly, at least 2×10^6 annihilation events were collection in each PALS histogram. The spectra were best fitted with three lifetime components. The first and the second lifetime (~ 250 ps and ~ 400 ps, respectively) are due to the short lived positrons from the sample, the Kapton seal and the copper, which are not relevant to the free volume of interest. The third

lifetime (τ_3 , 1.2 – 1.7 ns) is solely due to the positronium (i.e., bound state of a positron and an electron) annihilation in the sample and can be directly correlated to free volume hole size using the well-accepted Tao-Eldrup model(20). All data reported represent the mean of at least two replicates and standard error is an average of 0.02 ns.

2.11 Thermal Activity Monitor (TAM) Characterization of Global Dynamics

The effect of the amino acids on the global dynamics of the rHSA:SUC:amino acid formulations was investigated by directly measuring enthalpy relaxation by isothermal calorimetry (TAM, model 2277, Thermometric, Sweden). Samples were prepared under low humidity conditions (< 3% RH) by gently breaking up the lyophilized cake and placing approximately 100 mg of sample into a stainless steel ampoule (5 cc), which was tightly sealed to avoid exposure to ambient moisture after removal from the dry bag. Crystalline glycine was used as the inert reference. The ampoules were loaded into the measurement position of the TAM chambers after temperature equilibrium in the pre-measurement position for 30 minutes. The calorimeter was controlled at 50°C and the heat flow was measured for a minimum of 80 hours.

The modified stretched exponential function (MSE) was fit to the data, as described previously(7). The MSE equation can be expressed as follows,

$$P = 277.8 \cdot \frac{\Delta H_r(\infty)}{\tau_0} \cdot \left(1 + \frac{\beta t}{\tau_1}\right) \cdot \left(1 + \frac{t}{\tau_1}\right)^{\beta-2} \cdot \exp\left[-\left(\frac{t}{\tau_0}\right) \left(1 + \frac{t}{\tau_1}\right)^{\beta-1}\right] \quad (9)$$

where P is the power (heat flow, μW) divided by the mass of the sample, and the time

is in hours. The value 277.8 is a result of unit conversion, τ_0 and τ_1 are empirical relaxation times and β is the stretched power. The value of $\Delta H_r(\infty) = (T_g - T)\Delta C_p$, was determined directly from DSC measurements. The parameters β , τ_0 and τ_1 were determined using regression analysis software with the determined parameters, the stretched relaxation time, τ_D^β , can be obtained from Equation 10.

$$\tau_D^\beta = \left(\tau_0^{1/\beta} \cdot \tau_1^{(\beta-1)/\beta} \right)^\beta \text{ (Equation 10)}$$

The value τ_D^β is equivalent to the Kohlrausch-Williams-Watts (KWW) τ^β , and is used as an indication of global molecular mobility in the solids because it is a more robust quantity and not sensitive to systematic errors caused by changes in relaxation time during the measurement process as compared to the effective KWW structural relaxation time, $\tau_D(21)$.

3. Results and Discussion

3.1 Results of Stability Data

After freeze-drying the rHSA formulations had an average of 0.2% soluble aggregation. The aggregation was the same for the formulations both with and without amino acids, indicating the addition of amino acids to the sucrose-based formulation did not alter the in-process degradation. The soluble aggregation measured for both rHSA:SUC:serine and rHSA:SUC:methionine formulations followed square root of time kinetics. The rate constant for aggregation was determined for each formulation by fitting Equation 7 to

the stability data (Figure 2) for time zero, two, and three months. The methionine formulations had the best stability at 1:1:0.1 (rHSA:SUC:methionine), while the best stability for the serine formulations occurred at 1:1:0.4 (rHSA:SUC:serine). This stability data is representative of what was found previously for these two formulations by comparing the stabilization ratios (R_s , Equation 8)(16).

3.2 Evaluation of Formulation T_g

The glass transition temperature (T_g) for each amino acid formulation was measured by modulated DSC and compared to the previously estimated T_g values (using the Fox Equation)(15, 16). The serine formulations showed a large discrepancy between the estimated and measured T_g values. Measured values for rHSA:SUC:serine 1:1:X ($X = 0.1, 0.4, 0.8$) were 84°C, 72°C, and 66°C, respectively; the estimated T_g values were 99°C, 94°C, and 89°C. However, there was no significant difference between the measured and estimated T_g values for the methionine formulations (rHSA:SUC:methionine 1:1:X $X = 0.1, 0.4$), which were 99°C for $X = 0.1$ and 98°C for $X = 0.4$. If T_g was indicative of stability, the formulations with serine should be much less stable than the formulation without any amino acid. Obviously this is not the case, and T_g , at least for the serine formulations, is not a valid predictor of stability.

3.3 Density and Free Volume

The measured density, calculated volume change on mixing ($\Delta v'_m$), and calculated apparent specific volume (Φ_v') values are shown in Table 1. For the calculations, densities of crystalline serine and crystalline methionine were used for the density of the

pure amino acids (densities of the glassy state are not available), and densities of the amorphous rHSA:SUC systems and the amorphous rHSA:SUC:amino acid systems were used for all mixtures. A negative change on mixing is associated with a loss in free volume of the rHSA:SUC system upon mixing(8). The only negative change on mixing was for the for the 1:1:0.1 rHSA:SUC:methionine formulation, which is the most stable methionine formulation studied but not the most stable formulation tested. However, the most stable serine formulation tested (1:1:0.4) had the most positive volume change on mixing. Therefore, it appears that loss of free volume is not the key variable determining stability of rHSA:SUC:amino acid formulations, at least for methionine and serine. However, we are using crystalline densities to evaluate specific volume of the pure amino acid; had we used amorphous densities, the volume changes on mixing would have all been shifted lower, perhaps moving more of the entries in Table 1 into the negative range. However, it does not seem likely that this shift would have changed the rank order of the volume changes on mixing for the systems studied, so the basic conclusion would remain the same; stability and volume change on mixing are not well correlated.

For apparent specific volume, a loss in free volume in the “solvent” (rHSA:SUC) caused by addition of “solute” (the amino acid) would result in the apparent specific volume of the amino acid being less than the specific volume of the pure amino acid. Neither amino acid series shows a significant trend of apparent specific volume with concentration of amino acid (Table 1), meaning in these systems, apparent specific volume and partial specific volume are essentially the same. The average apparent

specific volumes for both the serine and methionine series are nearly equal to the pure crystalline serine and methionine specific volumes. Had we used amorphous densities to evaluate the specific volumes of the amino acids, the specific volumes of the pure amino acids would likely have been less than the corresponding apparent specific volumes in the mixtures, suggesting loss of free volume on forming the mixture. However, the trend of apparent specific volume minus the specific volume of the pure amino acid would not have correlated with the stability data. Therefore, changes in free volume, if any, appear to be minimal and while experimental uncertainty in the volumes of mixing and in the apparent specific volumes may obscure minor trends, there does not appear to be a significant meaningful correlation with stability. A weak correlation between volume changes on mixing and stability does exist, suggesting a decrease in rate constant as the $\Delta v'_{\text{mix}}$ becomes larger (more positive), however, it is the wrong direction for impact of an increase in free volume. Alternately, the apparent specific volumes show no significant concentration dependence above uncertainty in the data, meaning there is absolutely no correlation with stability data. It seems clear that changes in stability arising from addition of these amino acids to the protein:sucrose matrix are not related to “free volume”, at least that as measured by density.

3.4 Comparison of Dynamics

Besides T_g and potential free volume changes evaluated from densities, other measures of mobility were also investigated in an attempt to understand the mechanism of stabilization involved in the addition of serine and methionine to the 1:1 rHSA:SUC formulation. The various physical parameters associated with mobility in amorphous

solids are compared for the serine (Figure 3) and methionine formulations (Figure 4). The grey bar represents the rate constant of stabilization ($\ln(k)$), shown in Figure 2. The striped bars denote the structural relaxation time constant ($\ln(\tau_D^\beta)$) measured at 50°C, representative of global motions. The addition of serine (Figure 3) to the sucrose-based protein formulations resulted in decreased $\ln(\tau_D^\beta)$, indicating an increase in global motions. For the methionine formulations, $\ln(\tau_D^\beta)$ did not change with composition. These results are similar to the trends obtained from the T_g measurements.

In Figures 3 and 4, the white bar represents the measurement of fast dynamics ($1/\langle u^2 \rangle$) at 50°C, that is the motions occurring in the solid on a nanosecond timescale at 50°C. A larger $1/\langle u^2 \rangle$ is representative of suppressed motions on a nanosecond timescale. For the 1:1:X rHSA:SUC methionine formulations (Figure 4), the mobility decreases in the order of $X = 0 > 0.1 > 0.4$, however, this does not correspond to the order of rate constant, which decreases in the order of $X = 0 > 0.4 > 0.1$ (Figure 4). With serine, the trends are somewhat different (Figure 3). The 1:1:0.4 rHSA:SUC:serine formulation has the lowest mobility on a nanosecond timescale (i.e., highest $1/\langle u^2 \rangle$) out of all the serine formulations studied, and in this case was also the most stable formulation (i.e., smaller $\ln(k)$). The order for the decrease in fast dynamics for 1:1:X rHSA:SUC:serine is $X = 0.4 > 0 > 0.8 > 0.1$ and the order of stabilization by the amino acid, $\ln(k)$ decreases as follow: $X = 0.1 < 0.8 < 0.1 < 0$. While the most stable formulation ($X=0.4$) is also the formulation of lowest “fast dynamics” mobility, there is no obvious trend between stability and mobility for the rest of the serine formulations.

The mean diameter size of the free volume holes is measured by the positron lifetime, τ_3 , determined by PALS for the serine formulations. A larger τ_3 means a longer positron lifetime, indicating greater hole size. In theory, a smaller τ_3 represents smaller free volume and better stability. In Figure 3, the mean diameter size of the free volume holes is represented as $1/\tau_3$, denoted by the dotted bars. An increase in $1/\tau_3$ indicates smaller free volume. The order of decreasing hole size is as follows: $X = 0 \approx 0.4 > 0.8 \approx 0.1$, where the formulation without any serine ($X = 0$) has the smallest $1/\tau_3$ and the $X = 0.1$ formulation had the largest value of $1/\tau_3$. This trend in hole size is different from the trends in rate constant and $\langle u^2 \rangle$ for fast dynamics. The reason for lack of agreement between the measures of molecular mobility, the free volume hole size, and the stability is not clear. A previous study of formulations containing small molecules (glycerol, sorbitol) in a polymer-disaccharide formulation found qualitative agreement between fast dynamics and decreased free volume(8).

It has been previously shown that there is a linear correlation between decreased mobility on a nanosecond timescale and increased stability(11) for a range of protein formulations. The authors of the study compiled a large data set and found a correlation between improved stability (both physical and chemical) and decreased mobility on a nanosecond timescale. For the data in our study, the natural log of the aggregation rate constant at 50°C was plotted against $(1/\langle u^2 \rangle)$, for the amino acid formulations at 50°C and compared with the results of the previous literature study (data not shown). The

data in this study did overlap the data points from the previous study. However, just considering the data in our study, there is not a strong correlation between this measure of fast dynamics and stability. However, compared to the range of rate constants and values of $(1/\langle u^2 \rangle)$ covered in the previous literature study, our data for amino acid additions to rHSA:SUC have a very limited range for each property. It does seem that for our data set, factors other than fast dynamics as measured by neutron scattering are involved in determining stability. Other factors might include strong binding to the protein to prevent aggregation prone structures from forming or perhaps even stabilizing the native structure during freezing and drying so that storage stability is being conducted on a more stable conformation. Unfortunately, attempting to obtain structural information on solid-state protein formulations containing significant levels of amino acids is problematic as amino acids interfere with the standard technique, FTIR analysis of the Amide I band. It is possible that the addition of amino acids would provide the same correlation with a larger study covering a much larger range of stabilities and values of $\langle u^2 \rangle$ (i.e., more amino acids, more proteins, other types of degradation).

3.5 Comparison with Sorbitol

Two measures of mobility for rHSA:SUC:sorbitol formulations were investigated, and the results of the physical parameters studied (fast dynamics and T_g) are shown in Figure 5 along with previously determined stability results(16), where the stabilization ratio (Rs) is represented by the gray bars. As previously shown, the sorbitol formulations have the best stability at rHSA:SUC:sorbitol 1:1:0.3. In Figure 5, the white bar is representative of the T_g ($T_g/150$) as measured by DSC. With the addition of sorbitol, the T_g did decrease

for each formulation, as expected, indicative of decreased structural relaxation. The striped bars represent the mobility measured by neutron scattering ($1/\langle u^2 \rangle$) at 50°C. The order of decreased mobility for the 1:1:X rHSA:SUC:sorbitol is as follows: $X = 0.1 < 0.3 < 0 < 0.5$, while the order of increasing stability is $X = 0 < 0.1 < 0.5 < 0.3$. Thus, there is no obvious correlation between the fast dynamics and the stability. However, there is indication that the addition of sorbitol did suppress the fast dynamics relative to the formulation without any sorbitol.

4. Conclusions

The stability of lyophilized sucrose-based rHSA formulations was greatly improved by the addition of amino acids. As expected, there was no correlation between global motions and stability. In some cases, a decrease in mobility as measured by fast dynamics was accompanied by improved stability. However, no systematic trend was observed. The improved stability for the amino acid formulations was also not well correlated with a decrease in the system free volume as measured by volume changes on mixing, apparent specific volume or PALS data. In general, amino acids can be used to further improve the long-term stability of lyophilized protein formulations, but it was not clear how fast dynamics, free volume and structural relaxation are related to stability, if at all. It may well be that other mechanistic factors dominate, such as interactions between the amino acid and the protein that tend to stabilize native structure during freezing, which in turn provides a structure less prone to aggregation, or “binding” to the protein surface such that partial unfolding in the solid state is impeded without dramatically impacting the measures of mobility we have investigated. Small

molecule additives may be vital to the suppression of fast-dynamics in order to obtain room temperature or higher storage stability, but without further study, details of the mechanism remain obscure.

5. Acknowledgements

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6. References

1. Wang B, Tchessalov S, Cicerone MT, Warne NW, Pikal MJ. Impact of Sucrose Level on Storage Stability of Proteins in Freeze-Dried Solids: II. Correlation of Aggregation Rate with Protein Structure and Molecular Mobility. *Journal of Pharmaceutical Sciences*. 2008;98(9):1-22.
2. Chang L, Pikal MJ. Mechanism of Protein Stabilization in the Solid State *J Pharm Sci*. 2009;98(9):2886-908.
3. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational Design of Stable Lyophilized Protein Formulations: Some Practical Advice. *Pharm Res*. 1997;14(8):969-75.
4. Pikal MJ, Rigsbee D, Roy ML, Galreadth D, Kovach KJ, Wang B, et al. Solid State Chemistry II: The Correlation of Storage Stability of Freeze-Dried Human Growth Hormone (hGH) with Structure and Dynamics in the Glassy Solid. *Journal of Pharmaceutical Sciences*. 2008;97(12):5106-21.
5. Pikal MJ, Dellerman KM, Roy ML, Riggin RM. The Effects of Formulation Variables on the Stability of Freeze-Dried Human Growth Hormone. *Pharm Research*. 1991;8(4):427-36.
6. Wang B, Cicerone MT, Aso Y, Pikal MJ. The Impact of Thermal Treatment on the Stability of Freeze-Dried Amorphous Pharmaceuticals: II. Aggregation in an IgG1 Fusion Protein. *J Pharm Sci*. 2010;99(2):683-700.
7. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang X, et al. Mechanism of Protein Stabilization by Sugars During Freeze-Drying and Storage: Native Structure Preservation, Specific Interaction, and/or Immobilized in a Glassy Matrix? *Journal of Pharmaceutical Sciences*. 2005;94(7):1427-44.

8. Chieng N, Cicerone MT, Zhong Q, Liu M, Pikal MJ. Characterization of Dynamics in Complex Lyophilized Formulations: II. Analysis of Density Variations in Terms of Glass Dynamics and Comparisons with Global Mobility, Fast Dynamics, and Positron Annihilation Lifetime Spectroscopy (PALS). 2013.
9. Yoshioka S, Aso Y. Correlations Between Molecular Mobility and Chemical Stability During Storage of Amorphous Pharmaceuticals. *Journal of Pharmaceutical Sciences*. 2006;96(5):960-81.
10. Simmons DS, Cicerone MT, Zhong Q, Tyagi M, Douglas JF. Generalized Localization Model of Relaxation in Glass-Forming Liquids. *Soft Matter*. 2012;8:11455-61.
11. Cicerone MT, Douglas JF. Beta-Relaxation Governs Protein Stability in Sugar-Glass Matrices. *Soft Matter*. 2012;8:2983-91.
12. Cicerone MT, Soles CL. Fast Dynamics and Stabilization of Proteins: Binary Glasses of Trehalose and Glycerol. *Biophysical Journal*. 2004;86:3836-45.
13. Yoshioka S, Miyazaki T, Aso Y, Kawanishi T. Significance of Local Mobility in Aggregation of Beta-Galactosidase Lyophilized with Trehalose, Sucrose or Stachyose. *Pharm Research*. 2007;24(9):1660-7.
14. Cohen MH, Turnbull D. Molecular Transport in Liquids and Glasses. *J Chem Physics*. 1959;31(5):1164-9.
15. Forney-Stevens KM, Bogner RH, Pikal MJ. Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: Screening of 15 Amino Acids in Two Model Protein Formulations. 2013.

16. Forney-Stevens KM, Bogner RH, Pikal MJ. Concentration Ranges of Amino Acids To Further Stabilize Lyophilized Sucrose/rHSA Formulations. 2013.
17. Chang L, Shepherd D, Sun J, Tang X, Pikal MJ. Effect of Sorbitol and Residual Moisture on the Stability of Lyophilized Antibodies: Implications for the Mechanism of Protein Stabilization in the Solid State. *Journal of Pharmaceutical Sciences*. 2005;94(7):1445-55.
18. Kikuchi T, Wang BS, Pikal MJ. High-Precision Absolute (True) Density Measurements on Hygroscopic Powders by Gas Pycnometry: Application to Determining Effects of Formulation and Process on Free Volume of Lyophilized Products. *Journal of Pharmaceutical Sciences*. 2011:1-7.
19. Azuah RT, Kneller LR, Qiu Y, Tregenna-Piggott PLW, Brown CM, Copley JRD, et al. DAVE: A Comprehensive Software Suite for the Reduction, Visualization, and Analysis of Low Energy Neutron Spectroscopic Data. *J Res Natl Inst Stan Technol*. 2009;114(6):341-58.
20. Tao SJ. Positronium Annihilation in Molecular Substances. *J Chem Phys*. 1972;56:5499-510.
21. Liu J, Rigsbee DR, Stoltz C, Pikal MJ. Dynamics of Pharmaceutical Amorphous Solids: The Study of Enthalpy Relaxation by Isothermal Microcalorimetry. *Journal of Pharmaceutical Sciences*. 2001;91:1853-62.
22. Averett D, Cicerone MT, Douglas JF, dePablo JJ. Fast Relaxation and Elasticity-Related Properties of Trehalose-Glycerol Mixtures. *Soft Matter*. 2012 (8):4936-45.

7. Tables

Table 1: Density (g/cc), V_{mixing} (cc/g), and Apparent Specific Volume (ϕ_v' , cc/g) for 1:1:X rHSA:SUC:methionine and 1:1:X rHSA:SUC:serine formulations. Density was measured directly using a He pycnometer. V_{mixing} was calculated from equation 2 and ϕ_v' calculated from Equation 3.

Sample Composition	Density g/cc (\pm std. error)	V_{mixing} cc/g (\pm 0.01)	Φ_v' g/cc (\pm std. error)
rHSA:sucrose:methionine 1:1:0	1.4434 (0.0006)	-	-
rHSA:sucrose:methionine 1:1:0.1	1.4419 (0.0090)	-0.0030	0.71 (0.09)
rHSA:sucrose:methionine 1:1:0.4	1.4132 (0.0021)	0.0017	0.78 (0.01)
Methionine (Crystalline)	1.2965 (0.0020)	-	0.77
rHSA:sucrose:serine 1:1:0	1.4434 (0.0006)	-	-
rHSA:sucrose:serine 1:1:0.1	1.4438 (0.0030)	0.0021	0.69 (0.03)
rHSA:sucrose:serine 1:1:0.4	1.441 (0.0070)	0.0091	0.70 (0.02)
rHSA:sucrose:serine 1:1:0.8	1.4577 (0.0042)	0.0068	0.67 (0.01)
Serine (Crystalline)	1.5500 (0.0060)	-	0.64

8. Figures

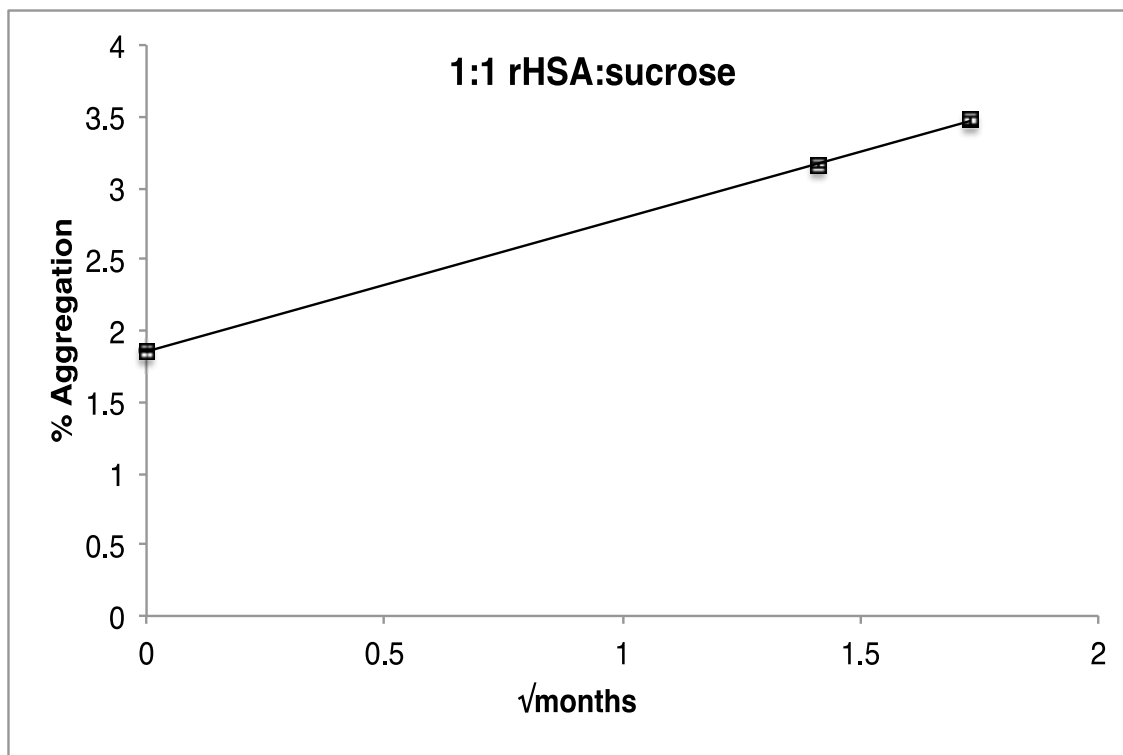


Figure 1: Illustration of square root of time kinetics for 1:1 rHSA:SUC formulation. $R^2 = 0.9999$ and the standard error of the slope is 0.013%. The three data points are from after freeze-drying (time zero) and after two and three months storage at 50°C. Error bars are smaller than symbols.

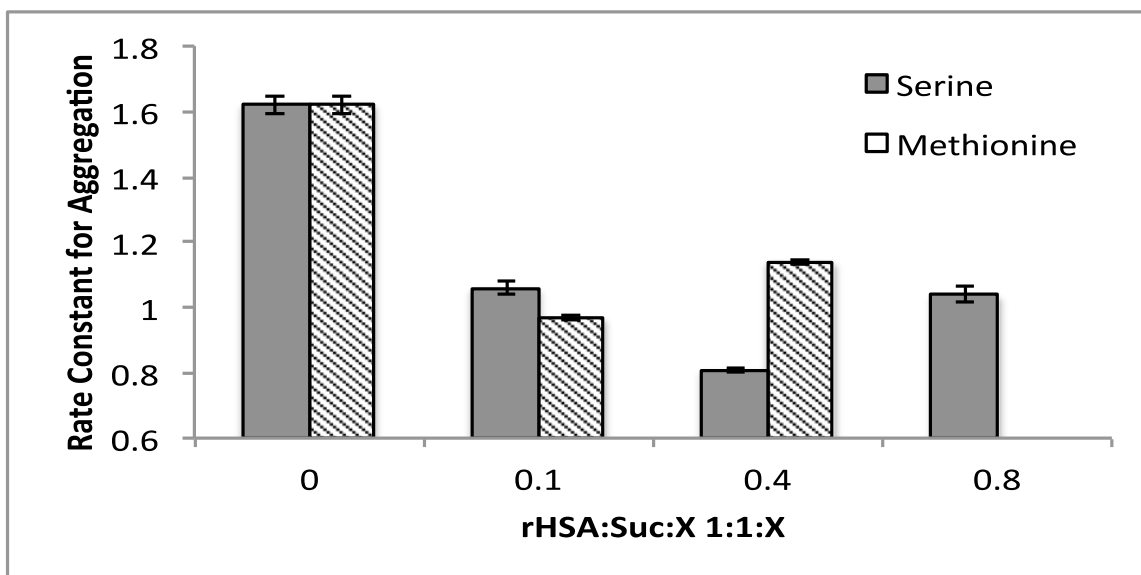


Figure 2: The rate constant for aggregation determined for 1:1:X (X = 0, 0.1, 0.4, 0.8) rHSA:SUC:serine and 1:1:X (X = 0, 0.1, 0.4)rHSA:SUC:methionine formulations. Rate constants were calculated from aggregation measured after processing (time zero) and after two and three months storage at 50°C.

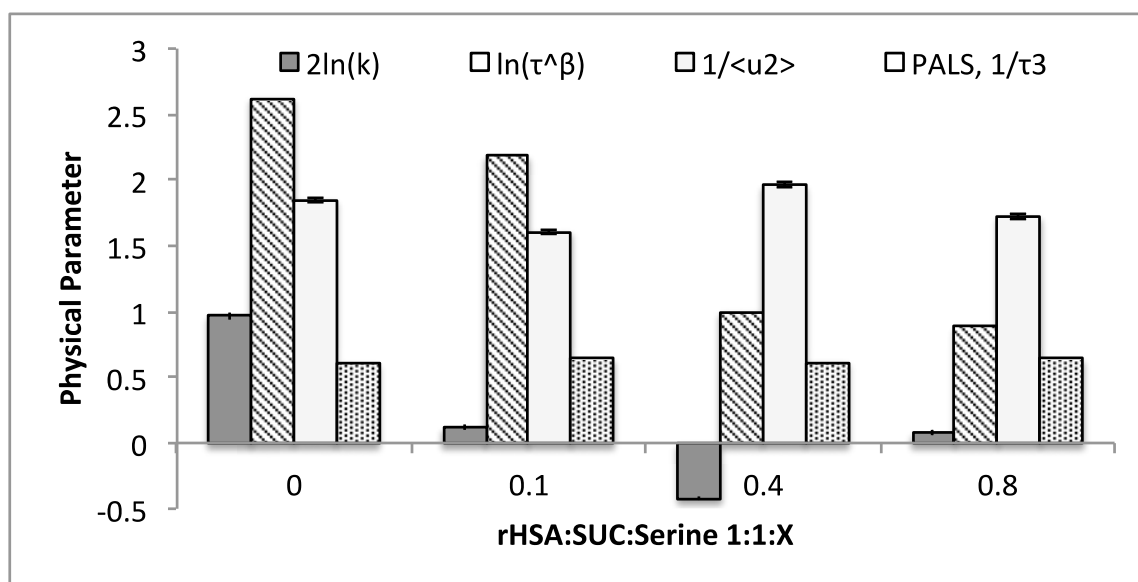


Figure 3: Aggregation rate constant (k) and measures of mobility for the 1:1:X ($X = 0, 0.1, 0.4, 0.8$) rHSA:SUC:serine formulations. $\ln(\tau^\beta)$ was determined by calorimetry and is a measure of global mobility, in hours. The value $1/\langle u^2 \rangle$ was determined from neutron backscattering and is a measure of fast dynamics. $\langle u^2 \rangle$ is the mean squared amplitude of motions (\AA). PALS, τ_3 is a measure of the mean diameter of “free volume holes”, in nanoseconds.

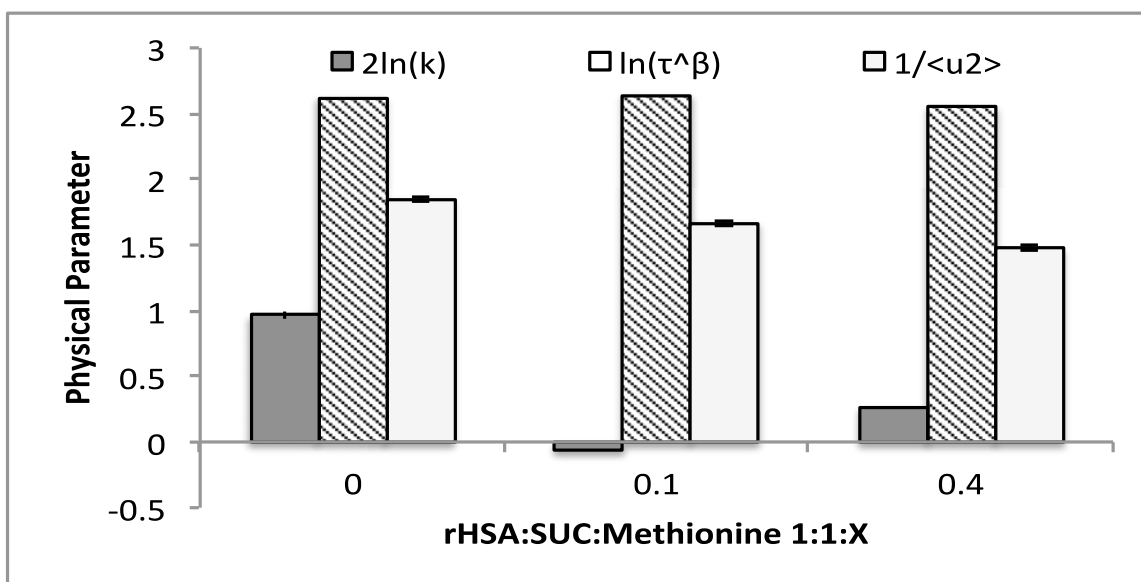


Figure 4: Aggregation rate constant (k) and measures of mobility for the 1:1:X ($X = 0, 0.1, 0.4$) rHSA:SUC:serine formulations. τ^β was determined by calorimetry and is a measure of global mobility, in hours. The value $1/\langle u^2 \rangle$ was determined from neutron backscattering and is a measure of fast dynamics. $\langle u^2 \rangle$ is the mean squared amplitude of motions (\AA).

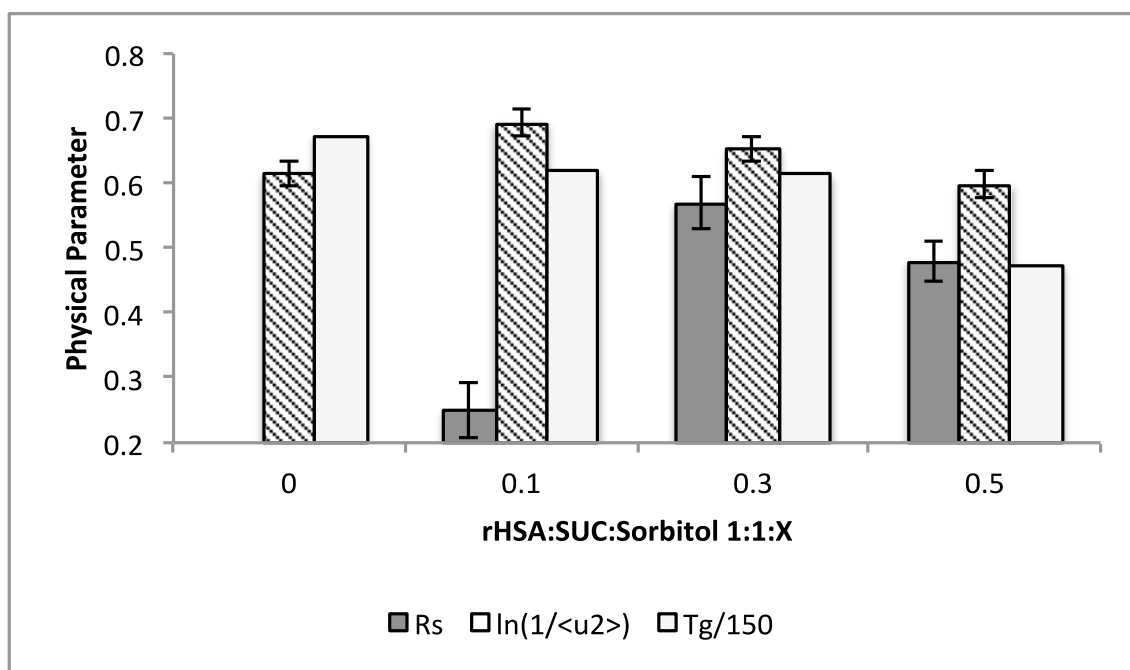


Figure 6: Stabilization ratio (R_s) for aggregation and measures of mobility for the 1:1:X ($X = 0, 0.1, 0.3, 0.5$) rHSA:SUC:sorbitol formulations. The stabilization ratio is taken from previous work (15, 16) and is calculated from aggregations assays after 3 months at 50. T_g ($^{\circ}\text{C}$) was determined by modulated DSC and is shown in the figure as $T_g/150$ to facilitate comparison with other data. The value $\ln(1/\langle u^2 \rangle)$ was determined from neutron backscattering and is a measure of fast dynamics, where $\langle u^2 \rangle$ is the mean squared amplitude of motions (\AA).

Chapter 6

Optimization of a Raman Microscopy Technique to Efficiently Detect Amorphous- Amorphous Phase Separation in Freeze-Dried Protein Formulations

Abstract

A confocal Raman microscopic technique was optimized to more efficiently detect amorphous-amorphous phase separation in freeze-dried protein formulations by assessing various instrument settings and sample preparation methods. The previous method required 2.5 hours/sample, while the optimized method only requires 0.5 hours/sample and is at least equivalent in ability to detect phase separation. Formulations containing an excipient and/or a protein were freeze-dried and Raman spectra were collected along 100-200 μm lines of each sample with a Renishaw Raman inVia confocal microscope using excitation by a 785-nm diode laser. Non-overlapping peaks representative of each component of interest were identified. At each point across the line map, the composition was evaluated from the intensity of the non-overlapping peaks. Phase separation was successfully detected using a large aperture, with a scan time of 5 seconds over a 200 μm line map and a 2 μm step size. Several protein formulations suspected of phase separation were evaluated for amorphous-amorphous phase separation. Phase separation was detected in the following formulations: Lysozyme-trehalose (1:1), lysozyme-isomaltose (1:1), β -lactoglobulin-dextran (1:1), β -lactoglobulin-dextran (1:3) and β -lactoglobulin-trehalose (1:1). Phase separation was not detected in Lysozyme-sucrose (1:1) and β -lactoglobulin-sucrose (1:1) formulations. The results are consistent with what was suggested in literature studies. The new optimized method successfully detected phase separation in several protein formulations, where phase separation was previously suspected and promises

to be a useful tool for detection of phase separation in amorphous therapeutic formulations.

1. Introduction

Therapeutic protein formulations are often freeze-dried to improve long-term storage stability. Still, the freeze-drying process imposes many stresses on the protein, potentially resulting in significant chemical and/or physical degradation. To minimize degradation during both process and storage, stabilizing excipients (e.g., disaccharides, polyols, polymers) are added to protein formulations(1). In order for the stabilizing excipients to successfully protect the protein from degradation, the excipients must be in the same phase as the protein. However, the freeze-drying process may lead to separation of the formulation matrix into several phases, resulting in protein-rich phase(s) and stabilizer-rich phase(s), where the protein is no longer fully protected by the stabilizer. Consequently, phase separation can affect product stability(2, 3).

Phase separation is thought to occur during the freezing step of the freeze-drying process, as a result of the low product temperature, formation of ice and resulting high concentration of the formulation components(4, 5). During freezing, the growth of ice crystals leaves a higher concentration of both the excipient(s) and protein in the interstitial space between the ice crystals. The protein and excipient(s) may physically separate into two distinct phases if the solute composition at the increased solute concentration is thermodynamically unfavorable and phase separation is not kinetically hindered(6, 7). The formation of multiple phases during the freezing step will either result in distinct crystalline phase(s) and/or amorphous phase(s) with less distinct phase boundaries.

Detection and characterization of multiple amorphous phases are keys to more fully understand how phase separation affects product stability. In the case of excipient crystallization, the crystalline phase can consistently be detected with techniques such as Powder X-Ray Diffraction (PXRD) or Polarized Light Microscopy (PLM). However, multiple amorphous phases are not as readily detectable. For example, two glass transition temperatures (T_g), characteristic of two amorphous phases, are not seen in phase separated lyophilized protein-excipient formulations due to a weak change in heat capacity and the broad temperature range of the glass transition of protein-rich systems. Alternatively, studies to detect phase separation using Scanning Electron Microscopy (SEM) are qualitative and often inconclusive(2, 8). As a result, amorphous-amorphous phase separation may go unidentified, potentially resulting in stability issues not being detected until well into long-term storage studies.

Recently, a Raman microscopic line mapping analysis was shown to be capable of detecting amorphous-amorphous phase separation in several binary polymer formulations(5) and a protein-disaccharide formulation(8). This analysis allows for chemical mapping of a sample to detect compositional fluctuations and quantifiably identify the presence of multiple amorphous phases. However, the previously reported analysis(5, 8) takes over 2.5 hours per sample, reducing its usefulness in routine screening for detection of multiple amorphous phases in therapeutic protein formulations.

Broadband Coherent Anti-Stokes Raman Scattering (BCARS) Microscopy was recently

compared to a typical confocal Raman microscopic technique in the analysis of multi-component formulations(9). BCARS acquired spectra 100 times faster, while achieving the same spatial resolution compared with confocal Raman spectroscopy. At the time of the present publication, this method, along with other Raman imaging instrumentation capable of rapid imaging(10) are becoming readily available for routine use. Ideally, another in depth analysis of phase separation should be conducted to evaluate the potential for these instruments to evaluate phase separation of amorphous formulations.

The objective of the present study was to further refine the Raman microscopic line mapping analysis for more routine use in the detection of amorphous-amorphous phase separation in freeze-dried protein formulations. The effects of sample preparation and various instrument settings on the efficiency and sensitivity of the analysis were evaluated. The newly optimized method was then used to analyze freeze-dried protein formulations where amorphous-amorphous phase separation was previously suspected based on either physicochemical characteristics(11) or long-term storage stability data(12).

2.Materials and Methods

2.1 Materials

Dextran of molecular weight 40,000 (DEX40K), polyvinylpyrrolidone of molecular weight 10,000 (PVP10K), D-mannitol (MAN) and sucrose (SUC) were purchased from Sigma (St. Louis, MO). Dextran of molecular weight range 4000-6000 (DEX5K) was purchased

from SERVA (Heidelberg, Germany). Isomaltose (IMT) was received as a gift from Seikagaku Baio, Bijinesu Co. (Tokyo, Japan). α,α -trehalose dihydrate (TRE) was purchased from Ferro Pfanstiehl (Waukegan, IL). All excipients were used as received.

Lysozyme (LYZ) from chicken egg white and β -lactoglobulin (BLG) from bovine milk were purchased from Sigma (St. Louis, MO). LYZ was dialyzed against either ultrapure water or 2mM phosphate buffer, pH 7.2 and BLG was dialyzed against 2mM phosphate buffer, pH 7.2, using a membrane with MWCO: 6-8,000 kDa (Spectrum Laboratories, Dominguez, CA). After dialysis the protein concentrations were confirmed by UV absorbance at 280 nm (Cary 50 Bio, Varian). The final concentrations of LYZ and BLG were 25 mg/mL and 10 mg/mL, respectively.

2.2 Methods

2.2.1 Sample Preparation

Solutions of MAN:SUC (1:2) were prepared at a total solute concentration of 100 mg/mL. Solutions of PVP:DEX5K (1:4 or 4:1 w/w) were prepared at a total solute concentration of 100 mg/mL. LYZ formulations were prepared at a 1:1 w/w ratio of LYZ:sugar (IMT, TRE, SUC) with a total solute concentration of 50 mg/mL. BLG:disaccharide (TRE, SUC) formulations (1:1 w/w) were prepared at a total solute concentration of 20 mg/mL. BLG:DEX40K formulations (1:1 or 1:3 w/w) were prepared with a total solute concentration of 20 mg/mL. Pure solutions of 50 mg/mL of DEX5K, DEX40K, PVP, MAN, SUC, TRE, IMT, 25 mg/mL LYZ and 10 mg/mL BLG were

prepared as controls.

2.2.2 Freeze-Drying

All solutions were filtered through 0.22 μm filters and filled in 1 mL volumes into 5 cc glass tubing vials and semi-stoppered with Daikyo Flurotech lyo stoppers (West Pharmaceutical, Lionville, PA). Freeze-drying was carried out using either a Durastop or LyoStar3 freeze dryer (FTS, SP Industries, Stone Ridge, New York). Thermocouples were placed in the bottom center of at least four vials per batch. After thermal equilibration at -5°C , all samples were cooled to -40°C at a rate of $1^{\circ}\text{C}/\text{min}$ during the freezing step.

Primary drying for PVP:DEX5K formulations was carried out at a shelf temperature of -18°C and a chamber pressure of 80 mTorr for at least 15 hours. For the SUC:MAN formulation, samples were annealed for 4 hours at a shelf temperature of -18°C to promote crystallization of the mannitol. After annealing the shelf was cooled to -40°C at a rate of $0.5^{\circ}\text{C}/\text{min}$ and held for 60 minutes. Primary drying was then carried out at a shelf temperature of -25°C and a chamber pressure of 80 mTorr for at least 20 hours. Primary drying for LYZ and BLG formulations was carried out at a shelf temperature of -29°C and a chamber pressure of 60 mTorr for at least 20 hours. The end of primary drying was taken as the time all product temperatures approached the shelf temperature, which indicates the end of primary drying in the thermocouple vials. Before moving to secondary drying, an additional “soak” time of 4 hours was used to ensure complete ice removal in all vials. In all cases product temperatures during primary

drying were maintained below collapse temperatures.

For secondary drying, the shelf temperature was increased at a rate of 1°C/min to 40°C and held for 6 hours. After cycle completion, vials were stoppered under partial vacuum (650 – 700 Torr), crimped with aluminum seals and stored at -20°C until analysis. All freeze-dried samples showed no visible signs of collapse. Water content was 0.5-1% (w/w) as determined by Karl-Fischer moisture titration (756 Coulometric Titrator, Metrohm, Riverview, FL). Crystallization of the MAN was confirmed by detection of birefringence with polarized light microscopy (50x objective).

2.2.3 Raman Mapping

Lyophilized cakes were gently broken up to remove them from the vial. Samples of each cake were either compressed in a 1/2" die using a Carver press at 5000 lb force for 1 min (as described previously)(5) or compressed manually using a bench top pellet press (Parr, Moline, IL) with a 1/8" die. Both methods produced pellets with a thickness greater than 0.25 mm and with surfaces sufficiently flat to allow confocal focusing with minimal changes in the sample height. The compressed samples were placed on a glass or a gold slide (both from Sigma, St. Louis, MO). Samples were exposed to ambient temperature and humidity during both sample preparation and data collection. Thus sorption of some water vapor was expected during handling and data acquisition (0.5 - 3 hours).

The slides were mounted on the motorized xyz stage of a Raman microscope (InVia,

Renishaw, Inc., Hoffman Estates, IL) equipped with confocal capability, a 785 nm diode laser (50 mW) and a 1200 L/mm grating. The instrument wavelength was calibrated with silicone at 520 cm^{-1} . Spectra from compressed pellets of pure components were obtained over a fixed window of 500 cm^{-1} and an accumulation time of 10 seconds. Spectra of each compressed sample were collected every $2\text{ }\mu\text{m}$ along a 100-200 μm line at the sample surface. Line maps were collected from triplicate pellets obtained from separate vials. The instrument was operated using either a small collection aperture ($20\text{ }\mu\text{m}$ slit, 2 pixel detector area) or a large collection aperture ($65\text{ }\mu\text{m}$ slit, 25 pixel detector area).

Each line map was analyzed as described previously (5, 8) using Thermo GRAMS/AI software (Thermo Fisher Scientific, Waltham, MA). Briefly, each spectrum was preprocessed by applying a Standard Normal Variate (SNV) algorithm. The SNV transformation minimizes variation due to sample height across the sample surface.(13) Baseline correction was not used, since there was minimal variation in fluorescence at the baseline, confirmed by overlay of spectra before the SNV was applied (data not shown).

Analytical peaks representative of each component of interest (i.e., protein or stabilizer) were identified.¹ Using the SNV transformed spectra, at each position, x_i , along the line map, the concentration of each component was determined from the intensity of the

¹ The identification of analytical peaks for each component is discussed in detail in section 3.1.

analytical peak, $I(x_i)$, where the intensity is proportional to the component concentration(5). The excess of a component, $r(x_i)$, relative to the average composition was then evaluated at each position along the line map, using Equation 1, where $\langle I(x) \rangle$ reflects the mean composition over the entire line map.

$$r(x_i) = \frac{I(x_i) - \langle I(x) \rangle}{\langle I(x) \rangle} \quad (1)$$

The relative excess of a component, $r(x_i)$ greater than zero indicates a higher than average concentration of a component at x_i , while a value of $r(x_i)$ less than zero is indicative of a lower than average concentration of that component at x_i .

The root mean square value (RMS) of the relative excess, r_{RMS} , provides a way to quantify the compositional deviation from the mean over the entire line map, Equation 2.

$$r_{RMS} = \sqrt{\frac{1}{n} \sum_{i=1}^n [r(x_i)]^2} \quad (2)$$

However, r_{RMS} is not zero even for compressed samples of pure components. The nonzero value of r_{RMS} for a pure component reflects residual variation in the Raman intensities that were not eliminated by SNV. The value of r_{RMS} of a phase-separated sample is expected to be significantly higher than that of a pure component, where the average r_{RMS} value of a pure component depends of the components physical properties and processing conditions. As described earlier (5), a sample was considered phase separated only when the difference between the sample r_{RMS} and the pure component

r_{RMS} was significant at a level of 0.10 (i.e., $p\text{-value} < 0.10$), using Student's t-Test. Thus, there is a 90% probability that the r_{RMS} is higher than that found due to normal variations and that the sample is then considered to be phase separated. The extent of phase separation can then be inferred from the magnitude of r_{RMS} and this value also provides a quantitative measure of the composition of the amorphous phase.

3. Results and Discussion

3.1 Identification of Analytical Peaks

A high intensity Raman peak for each individual component that did not overlap with peaks of the other component in the binary formulation, was selected as the analytical peak for that component of interest (Figure 1). Analytical peaks for PVP and DEX5K were identified at 935 and 541 cm^{-1} , respectively (Figure 1a). For SUC and MAN, analytical peaks were identified at 833 and 875 cm^{-1} (Figure 1b).

In the case of the formulations that contained protein, non-overlapping peaks suitable for analysis of composition for the excipient could not be identified (within the fixed window scan range of 500 cm^{-1}). However, there were suitable peaks that did not overlap excipient peaks at 759 cm^{-1} for LYZ (Figure 1c) and 1655 cm^{-1} for BLG (Figure 1d). In protein formulations, separation of the protein from the stabilizer into a protein-rich phase, will lead to a lower stabilizer concentration near the protein, perhaps resulting in poor stability. Therefore, detection of protein-rich phases was most relevant for the determination of potentially detrimental phase separation relevant to protein

stability. The analytical protein peak was found to be sufficient to identify protein-rich phases.

3.2 Evaluation and Optimization of Instrument Parameters and Sample Preparation

3.2.1 Spectral Accumulation Time

Spectral accumulation time is the period the sample is exposed to incident light and the detector collects photons. A longer accumulation time results in higher peak intensity (Figure 2). A short accumulation time results in shorter experimental time and lower exposure of the sample to the laser. Particularly for samples with low component concentrations, a longer accumulation time is needed to obtain sufficient peak intensities and to overcome spectral noise. However, longer accumulation times are not universally better since detector saturation can occur, resulting in peak distortion. The appropriate accumulation time is highly dependent on the collection aperture, discussed in the next section.²

3.2.2 Collection Aperture

Confocal microscopes are designed to collect light emanating from a small sample depth, by using a small aperture through which light passes to reach the detector. As a result, the majority of light that is analyzed by the detector comes from the plane of

² Longer accumulation times require increased exposure of the sample to the laser. For the accumulations times used and the samples used in this study, there was no discernible damage at the surface due to heat or light from the laser. However, such effects should ideally be evaluated for each sample.

focus. The collection aperture (i.e., slit width) along with the focal objective regulates the depth above and below the focal plane from which light is collected and the area of the focal plane analyzed by the detector. A large aperture (65 μm slit, 25 pixel detector) collects light from a greater depth and larger area of focus, resulting in higher peak intensity. Higher peaks provide larger spectral signal-to-noise ratio (S/N) due to the large amount of light collected. However, both the large focal depth and large focal area also lead lower spatial resolution in both the z-direction and the xy-plane, respectively. Conversely, a small aperture (20 μm slit, 2 pixel detector area) collects less light from above and below the plane of focus, resulting in higher spatial resolution in the z-direction and a smaller area analyzed by the detector, but also results in lower spectral S/N(14).³

The raw peak intensity increases with increasing accumulation time for both apertures, with at least a two-fold increase in peak intensity for every 5 second increase in accumulation time (Figure 2). A large collection aperture allows a much larger amount of light to be collected reducing the accumulation time required to achieve the same spectral S/N as a small collection aperture. The use of the larger aperture results in improved spectral sensitivity but also a lower spatial resolution in the z-direction and the xy-plane.

A spectrum is collected at each point across a line on the sample surface and each

³ Both the sample volume illuminated by the laser and the area imaged onto the detector are dependent on the geometrical optics of the instrument and the elastic scatter by the sample and will impact the S/N.

spectrum collected is representative of the composition within the sample volume from which the light is collected. In a phase-separated sample, it is possible for light to be collected from more than one phase if two phases or a phase boundary is within the sample volume. Light collected through the larger aperture size has a higher probability of reporting the average of several phase-separated domains; this is particularly true in the case of small domain sizes. Therefore, a phase-separated sample may appear to be homogeneous when the domain sizes are small relative to the analyzed xy-area and the focal depth in the z-direction. In order to detect smaller domains, a small aperture size can be used to improve the spatial resolution. However, a long accumulation time would be necessary to achieve sufficiently high peak intensities to obtain adequate spectral S/N (e.g., 60 seconds was used in the previously reported method(5)).

Due to the possibility of collecting light from multiple domains at any position along the line, analysis of Raman line maps can never confirm that a sample is homogeneous; however, it can quantifiably confirm the presence of more than one phase. If the r_{RMS} of a component from the mapping analysis is sufficiently larger than that of the r_{RMS} of the pure component to statistically confirm phase separation, using the larger aperture(5), then the use of a small aperture will not contradict the findings. However, the use of a small aperture may increase the r_{RMS} allowing identification of small phases, not possible with the larger aperture constraints. To minimize sample collection time, a larger aperture with a short accumulation time can be used first, where smaller apertures and longer collection times are reserved for samples not found to be phase separated by the shorter experiments.

3.2.4 Line Map Length

Line mapping was utilized rather than area mapping to minimize data collection time, whereas area mapping can take up to a full day for one sample.⁽⁵⁾ The length of the line defines the sampling size and the SNV applied to the raw data transforms the spectrum such that the mean Raman intensity along the map is zero with a unit standard deviation. The sample studied must be large enough to obtain a mean intensity that corresponds to the average composition⁽¹⁵⁾. The optimal length of the line map under analysis is also a compromise between the domain sizes of the sample being evaluated and experimental time. If the domain sizes are large, a longer line map is needed to sample an adequate number of phases in order to fully characterize the sample and report the true average composition ($\langle I(x_i) \rangle$).

To quantitatively evaluate the effect of line map length on the root mean square relative excess of each component (r_{RMS}), a formulation (1:2 MAN:SUC) with known phase separation was studied. After annealing during the freeze-drying process, mannitol is known to crystallize, leaving a sucrose-rich phase and a pure mannitol phase.⁽¹⁶⁾ For this study, crystallization was confirmed by the presence of birefringence using polarized light microscopy. Line maps of 200 μm were collected from the phase separated 1:2 MAN:SUC formulation. The r_{RMS} values for the 100 μm line maps (0-100 μm and 100 - 200 μm) and 200 μm line maps (0 – 200 μm), were compared (Table 1). The average r_{RMS} for the two line map lengths were not significantly different. However, the ability to detect phase separation from the 200 μm line map was greater than from the 100 μm line map due to the lower relative standard deviation of r_{RMS} values from the

longer line maps. For SUC, the relative standard deviation substantially decreased for the 200 μm line maps, compared to the 100 μm line maps, while the relative standard deviation for MAN was not significantly different between the two line map sizes. This difference is due to the nature of the composition of the phase. Mannitol is a component of both phases, while sucrose is only found to be in the amorphous phase. The value of r_{RMS} and the ability to detect phase separation depends in part on the compositional difference in the phases. A large difference in phase composition favors detection of phase separation.

While domain sizes were not directly measured, an effort to estimate domain sizes in phase-separated systems was attempted from inspection of the normalized line-maps. Raman peak intensities for both SUC and MAN were normalized to the average intensity of the corresponding component across a 200 μm line map (Figure 3). Fluctuations in the normalized peak intensity for each component along the line map demonstrate how the composition changes with distance in the sample. For example, at 20 μm into the line map, there is a clear MAN phase, where the MAN intensity is much greater than the SUC intensity, while at 40 μm along the line map, there is a SUC-rich phase. The size of the MAN phases across the line map is about 20 μm , while the SUC-rich domains vary between 10 - 40 μm . The apparent uniformity of the MAN phases may be due to the inherent order of the crystalline state and corresponding crystal size, compared to the less well defined amorphous state.

For the first half of the map (0 – 100 μm) shown in Figure 3, the fluctuations in

composition between phases are much greater than the standard deviations of the pure components, indicated by the horizontal lines at ± 0.05 . However, between 100 and 200 μm , the deviations are less pronounced, and some of the peak intensities are not significantly different than the average peak intensity. This qualitatively shows how the relative excess of a component across the line map is highly variable, depending on compositional difference and size of the phase domains.

Figure 3 shows the benefit of using a longer line map, particularly with the larger aperture. Using the larger aperture and shorter exposure time, the 200 μm line map is able to assess a larger number of domains, thereby providing a more accurate evaluation of the average compositional fluctuations and provide a better estimate of r_{RMS} . Most likely domain size is not known before conducting the experiment; therefore, choosing a 200 μm line map over a 100 μm will help to ensure a sufficient number of domains are studied.

3.2.5 Sample Compression

Surface roughness makes it difficult to maintain collection from the same sample thickness (z-direction). A flat surface improves focusing along the entire line map, thereby minimizing artifacts due to sample height variation. In the original Raman microscopic method, the lyophilized cake was compressed into a pellet by a Carver Press to obtain a sample with a sufficiently flat surface⁽⁵⁾. In the present study, no difference was found between the carefully controlled compression⁽⁵⁾ method and the more accessible manual compression method (data not shown). Therefore, the simplest

means available can be used to prepare the sample, as long as the sample pellet has a adequately flat surface for focusing and there is no damage to the sample during preparation.

3.3 Comparison of Optimized Method with Original Method

The optimized parameters are summarized in Table 2 and compared with the previously reported experimental design(5). We note that the previous method used only a glass microscope slide for analysis. Both a glass and a gold slide were compared for this study and no significant difference in the detection of phase separation was found.

The ability of the optimized method to identify phase separation was compared to the results from the previous Raman microscopic study using a binary-polymer formulation (PVP and DEX5K), shown in Table 3(5). Previously, the formulation of 1:4 PVP:DEX5K was confirmed to be phase separated by both DSC (presence of two T_g ' values) and by the original Raman microscopic method. The formulation of 4:1 PVP:DEX5K was not found to be phase separated, as detected by both DSC and the Raman microscopic method. The optimized Raman method confirmed phase separation in the 1:4 PVP:DEX5K formulation at a p -value < 0.10 for both the DEX5K and PVP components. Additionally, like the original method, the optimized method did not detect phase separation for the 4:1 PVP:DEX5K formulation at a p -value < 0.10 . Based on this comparison, the methods are equivalent in their ability to detect phase separation. However, the optimized method allows for all four PVP:DEX5K samples to be analyzed in less time (2 hours) required to previously analyze one sample (2.5 hours).

3.4 Evaluation of Formulations Suspected of Phase Separation

Two sets of protein formulation studies suggested to be (but not confirmed to be) phase separated were analyzed using the newly optimized Raman microscopic technique. Phase separation was proposed as a potential explanation for solid-state NMR relaxation times of formulations of lysozyme (LYZ) in isomaltose (IM) and in trehalose (TRE)(11). In a separate report, phase separation of β -lactoglobulin (BLG) and dextran 40K (DEX40K) formulations was hypothesized due to the decreased stability after high temperature storage(12). Here, the new optimized Raman microscopic analysis is used to examine the formulations in each series for phase separation identification and where possible to further interpret the presence of phase separation as it relates to formulation physical properties and storage stability.

3.4.1 Evaluation of LYZ Formulations

In previous research the effects of trehalose (TRE), sucrose (SUC) and isomaltose (IM) on the molecular motions of freeze-dried lysozyme (LYZ) were investigated(11). All stabilizing sugars were formulated at a 1:1 (w/w) ratio of LYZ:Sugar. The relaxation time constant (τ_c) for the LYZ carbonyl carbons was determined by analysis of $T_{1\rho}$ relaxation times obtained from solid-state NMR spectroscopy. In this study, two τ_c values were identified, corresponding to relaxation occurring on two different timescales (β -relaxation and γ -relaxation). A larger τ_c corresponds to a longer relaxation time indicating slower mobility. Larger relaxation time constants have been correlated to improved stability where it is thought that the mobility of the protein is inhibited by the sugar matrix.

For this study, $\tau_c(25^\circ\text{C})$ values for LYZ:Sugar formulations were compared to the $\tau_c(25^\circ\text{C})$ value determined for pure LYZ for both β -relaxation and γ -relaxation timescales. Comparing the τ_c values for β -relaxation, it was found that LYZ had decreased mobility in the presence of SUC, had slightly increased mobility in the presence of TRE and had much higher mobility in the presence of IM (Table 4). The τ_c corresponding to γ -relaxation showed no change in mobility of LYZ in the presence of any of the sugars(11). Phase separation of the LYZ:IM formulation and potentially in the LYZ:TRE formulation was postulated to explain the decrease in τ_c for the β -relaxation timescale. This decrease in τ_c may result from the formation of a LYZ-rich phase and a IM-rich phase or TRE-rich phase, where the IM and TRE are not at a high enough concentration to suppress the β -relaxation of the LYZ-rich/sugar-poor domains but sufficient to suppress relaxation on the γ -relaxation timescale. It is not clear why τ_c for β -relaxation of LYZ in IM and in TRE is less than the τ_c for pure LYZ.

To investigate phase separation in this series of formulations, LYZ:TRE, LYZ:SUC and LYZ:IM at 1:1 w/w ratio were evaluated using the optimized Raman microscopic method (Table 4). LYZ:IM had a larger r_{RMS} than the LYZ:TRE formulation, indicating a higher degree of phase separation for the LYZ:IM formulation, in agreement with the τ_c value corresponding to the β -relaxation timescale. The phase separated formulations correlate to a decrease in τ_c for both LYZ:TRE and LYZ:IM formulations, indicating the formation of LYZ-rich and sugar-rich phases. The previous Raman study also confirmed phase separation in a 1:1 (w/w) LYZ:TRE formulation(8).

Phase separation can be qualitatively evaluated by examining the normalized peak intensity of LYZ over the 200 μm line map for each formulation (Figure 4). For LYZ:SUC formulations, the LYZ normalized peak intensity values do not deviate outside of the natural fluctuations seen for pure LYZ (Figure 4a). For the LYZ:TRE (Figure 4b) and LYZ:IM (Figure 4c) formulations, this is not the case. The LYZ peak values fluctuate out of the range of one standard deviation. The 1:1 LYZ:IM line map (Figure 4c) shows greater fluctuations in normalized intensity than does the 1:1 LYZ:TRE line map (Figure 4b). Both line maps suggest small domains (small relative to the MAN:SUC system in Figure 3) that are protein-rich and protein-poor. The larger deviations in the 1:1 LYZ:IM line map are consistent with the large value of r_{RMS} and lower τ_c of lysozyme. The greater fluctuations suggest a greater compositional variation between the phases in LYZ:IM and LYZ:TRE.

3.4.2 Evaluation of BLG Formulations

The long-term storage stability of freeze-dried β -lactoglobulin (BLG) formulated with sucrose (SUC), trehalose (TRE) or dextran 40K (DEX40K) at several weight ratios was previously evaluated by measuring aggregation at 70°C; however only two concentrations are considered for the Raman experiments(12). The rate constant for aggregation decreased with increasing concentrations of SUC and TRE. However, the aggregation rate constants for formulations of BLG:DEX40K were higher than the rate constant for BLG aggregation in the disaccharide formulations (Table 5). Since there was no crystallization of DEX40K (detected by polarized light microscopy), amorphous-amorphous phase separation was suggested as one possible cause of the increase in

aggregation rate for the BLG:DEX40K formulations(12). DSC studies have also suggested phase separation in other dextran-based protein formulations(17, 18).

The newly optimized Raman microscopic method was used to evaluate the possible amorphous-amorphous phase separation in these formulations. Due to the lower BLG concentration in the 1:3 BLG:DEX40K formulation, the intensity of the BLG peak was too weak using a 5 second accumulation time. Instead, an accumulation time of 10 seconds was used to produce acceptable peak intensities.

Phase separation was identified in both the 1:1 and 1:3 BLG:DEX40K formulations, but not detected in the 1:1 BLG:SUC formulation (Table 5). Unexpectedly, phase separation was detected in the 1:1 BLG:TRE formulation, even though there was no increase in the aggregation rate constant. The r_{RMS} values for the DEX40K formulations double the value r_{RMS} for pure BLG, potentially indicating a large difference in the composition of each phase. In contrast, the TRE formulation had a value of r_{RMS} close to that of the r_{RMS} for pure BLG. Qualitatively, the extent of phase separation can be seen in Figure 5 from the normalized peak intensities over the length of the line map. The line map of the BLG:SUC formulation (Figure 5a) shows little phase separation, with the BLG intensities remaining within the standard deviation for normal fluctuations of intensities. The BLG:TRE (Figure 5b) formulation and both BLG:DEX40K (Figure 5c, 5d) formulations show greater fluctuations over the length of the line map, indicative of phase separation. The BLG:TRE formulation has larger domains with fewer regions of very high BLG intensity along the line map compared with both of the BLG:DEX40K

formulations.

The data are consistent with lower compositional deviation in the BLG:TRE formulation. At a 1:1 w/w ratio for the BLG:TRE formulation, there may be sufficient concentration of trehalose present in the BLG-rich phase to effectively protect the protein against aggregation. Even over the smaller line map for the 1:3 BLG:DEX40K formulation, phase separation is apparent, with large deviations along the map, compared with the 1:1 BLG:DEX40K formulation.

The presence of DEX40K seems to increase the rate constant of aggregation for the BLG formulation, compared to the BLG formulation without any additive. The increase in aggregation may be a result of the formation of two amorphous phases. It is not clear whether the BLG-rich phase or the DEX40K-rich phase contributes to increased aggregation. One may speculate that the DEX40K-rich phase enhances the aggregation rate of the BLG remaining in that phase, since the aggregation rate is larger than the aggregation rate of pure BLG. A potential mechanism of destabilization by dextran is its higher apparent acidity in the freeze-dried form, as was observed by measuring the Hammett acidity function and acceleration of acid-catalyzed inversion of sucrose in the freeze-dried formulations(19).

4. Summary and Conclusions

A Raman microscopic technique was optimized to more efficiently evaluate freeze-dried protein formulations for amorphous-amorphous phase separation. By optimizing

spectral accumulation time, collection aperture size and map length, the total experimental time was significantly reduced. The optimized method was five times faster than the previously described method. In addition to the more quantitative evaluation of phase separation using r_{RMS} values, qualitative interpretation of the line maps can provide some indication of domain size and compositional deviation between the phases.

The new method correctly assessed phase separation in model systems (PVP:DEX5K, MAN:SUC) known to phase separate. Phase separation was also detected in samples in which phase separation was suggested (BLG:DEX40, LYZ:IM, LYZ:TRE) but not confirmed. Confirmation of phase separation provided further insight with which to interpret the previously reported data. The new method also detected phase separation in a formulation where phase separation was not suspected (BLG:TRE). The systems investigated in this study involved only binary phase formulations, but the same method could potentially be useful in detecting multiple amorphous phases.

The optimized Raman method promises to be a useful tool for detection of phase separation in amorphous therapeutic formulations. Potentially, this method may be used as an early indicator of suboptimal stability due to phase separation without the need for long stability studies.

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6. References

1. Singh SK. Impact of Product-Related Factors on Immunogenicity of Biotherapeutics. *J Pharm Sci.* 2011;100(2):354-87.
2. Padilla AM, Pikal MJ. Phase Separation of Freeze-Dried Amorphous Solids: The Occurrence and Detection of Multiple Amorphous Phases in Pharmaceutical Systems. In: Rey L, May JC, editors. *Freeze-Drying/Lyophilization of Pharmaceuticals and Biological Products* New York: Informa Healthcare; 2010.
3. Pikal MJ, Rigsbee D, Roy ML, Galreadth D, Kovach KJ, Wang B, et al. Solid State Chemistry II: The Correlation of Storage Stability of Freeze-Dried Human Growth Hormone (hGH) with Structure and Dynamics in the Glassy Solid. *Journal of Pharmaceutical Sciences.* 2008;97(12):5106-21.
4. Franks F. Cryobiochemistry- Responses of Proteins to Suboptimal Temperatures. *Biophysics and Biochemistry at Low Temperatures.* Cambridge, UK: Cambridge University Press; 1985.
5. Padilla AM, Ivanisevic I, Yang Y, Engres D, Bogner RH, Pikal MJ. The Study of Phase Separation in Amorphous Freeze-Dried Systems. Part 1: Raman Mapping and Computation Analysis of XRPD Data in Model Polymer Systems. *Journal of Pharmaceutical Sciences.* 2010;100(1):206-22.
6. Izutsu K-i, Kojima S. Freeze-Concentration Separates Proteins and Polymer Excipients into Different Amorphous Phases. *Pharm Res.* 2000;17(10):1316-22.
7. Izutsu K-i, Yoshioka S, Kojima S, Randolph TW, Carpenter JF. Effects of Sugars and Polymers on Crystallization of Poly(ethylene glycol) in Frozen Solutions: Phase Separation Between Incompatible Polymers. *Pharm Res.* 1996;13(9):1393-400.

8. Padilla AM, Pikal MJ. The Study of Phase Separation in Amorphous Freeze-Dried Systems, Part 2: Investigation of Raman Mapping as a Tool for Studying Amorphous Phase Separation in Freeze-Dried Protein Formulations. *Journal of Pharmaceutical Sciences*. 2010;100(4):1467-74.
9. Hartshorn CM, Lee YJ, Canfield N, Heddlestone J, Liu Z, Camp CH, et al. Multicomponent Chemical Imaging of Pharmaceutical Solid Dosage Forms with Broadband CARS Microscopy. 2012.
10. Combining Fast Raman Imaging with Optimization of the Spatial Resolution for Complete Measurement. In: HORIBA Jobin Yvon I, editor. *The Application Notebook: Molecular Spectroscopy*; 2008.
11. Yoshioka S, Forney KM, Aso Y, Pikal MJ. Effect of Sugars on the Molecular Motion of Freeze-Dried Protein Formulations Reflected by NMR Relaxation Times. *Pharm Res*. 2011;28(12):3237-47.
12. Perez-Moral N, Adnet C, Noel TR, Parker R. The Aggregative Stability of Beta-Lactoglobulin in Glass Mixtures with Sucrose, Trehalose and Dextran. *Eur J Pharm Biopharm*. 2011;78(2):264-70.
13. Barnes RJ, Dhanoa MS, Lister S. Standard Normal Variate Transformation and De-Trending of Near-Infrared Diffuse Reflectance Spectra. *Appl Spectrosc*. 1989;43:772-7.
14. Sheppard CJR, Shotton DM. *Confocal Laser Scanning Microscopy*. New York: Springer-Verlag; 1997. 106 p.
15. Zhang L, Henson MJ, Sekulic SS. Multivariate Data Analysis for Raman Imaging of a Model Pharmaceutical Tablet. *Analystica Chimica Acta*. 2005;545:262-78.

16. Sundaramurthi P, Patapoff TW, Suryanarayanan R. Crystallization of Trehalose in Frozen Solutions and its Phase Behavior during Drying. *Pharm Res.* 2010;27:2374-83.
17. Heller MC, Carpenter JF, Randolph TW. Conformational Stability of Lyophilized PEGylated Proteins in a Phase-Separating System. *Journal of Pharmaceutical Sciences.* 1999;88:58-64.
18. Schaink HM, Smit JAM. Protein-Polysaccharide Interactions: The Determination of the Osmotic Second Virial Coefficients in Aqueous Solutions of Beta-Lactoglobulin and Dextran. *Food Hydrocolloids.* 1993;21:1389-96.
19. Lu E, Ewing S, Gatlin L, Suryanarayanan R, Shalaev E. The Effect of Bulking Agents on the Chemical Stability of Acti-Sensitive Compounds in Freeze-Dried Formulations: Sucrose Inversion Study. *J Pharm Sci.* 2009;98(9):3387-96.

7. Tables

Table 1: Comparison of r_{RMS} values for 100 μm line maps and 200 μm line maps based on peak intensity at 815 cm^{-1} (MAN) and 833 cm^{-1} (SUC) for a 1:2 (w:w) MAN:SUC formulation.

		Root Mean Square of the Relative Excess of Each Component, r_{RMS} *					
		MAN			SUC		
Line Map		100 μm		200 μm	100 μm		200 μm
		0-100	100-200	0-200	0-100	100-200	0-200
No.	1	0.116	0.071	0.110	0.479	0.174	0.366
	2	0.043	0.040	0.056	0.300	0.243	0.276
	3	0.296	0.076	0.260	0.753	0.220	0.517
	4	0.065	0.047	0.057	0.189	0.189	0.266
Average r_{RMS}		0.094		0.121	0.318		0.356
Rel. Std. Dev. (%)		90		80	63		33

* r_{RMS} calculated using Equation 2

** r_{RMS} for pure MAN = 0.023 and r_{RMS} for pure SUC = 0.021 (n = 4)

Table 2: Summary of optimized method parameters compared with the original method parameters (5).

Parameters	Original Method	Optimized Method
Line Map Length	100 μm	200 μm
Aperture Setting	20 μm	65 μm
Accumulation Time	60 sec	5 sec
Collection Time	2.5 hours	0.5 hours

Table 3: Comparison of the optimized method with the original method (5) to distinguish phase separation in 1:1 and 4:1 (w:w) PVP:DEX5K formulations from statistical difference between r_{RMS} values of mixtures and r_{RMS} values of pure components.

Formulation	Component of Interest	Original Method			Optimized Method		
		r_{RMS}		p-value**	r_{RMS}		p-value
		Pure Component	Mixture		Pure Component	Mixture	
PVP:DEX5K 1:4	PVP	0.016 (0.002)*	0.037 (0.003)	0.003***	0.022 (0.055)	0.094 (0.011)	0.004
	DEX5K	0.017 (0.001)	0.028 (0.005)	0.031	0.016 (0.056)	0.024 (0.005)	0.055
PVP:DEX5K 4:1	PVP	0.016 (0.002)	0.025 (0.010)	0.130	0.022 (0.055)	0.028 (0.006)	0.113
	DEX5K	0.017 (0.001)	0.008 (0.002)	0.992	0.016 (0.056)	0.014 (0.003)	0.816

*Values in parentheses are standard errors (n = 3)

**p-value evaluated for the difference between the r_{RMS} of the component in the mixture and the r_{RMS} of the pure component

*** Bold values represent phase separation based on p-value < 0.10

Table 4: Comparison of the relaxation time constants (τ_c), r_{RMS} values and p-values for the LYZ formulations to distinguish phase separation.

Formulation	β -Relaxation τ_c (μ s) (25°C)	γ -Relaxation τ_c (μ s) (25°C)	r_{RMS} (Std. Error)	p-value*	Phase Separation Detected*
Pure LYZ	100	0.3	0.051 (0.024)	-	-
1:1 LYZ:SUC	200	0.3	0.043 (0.008)	0.849	NO
1:1 LYZ:TRE	70	0.3	0.076 (0.021)	0.087	YES
1:1 LYZ:IM	20	0.3	0.097 (0.031)	0.064	YES

*p-value evaluated from the difference between the r_{RMS} LYZ in the mixture and the r_{RMS} of the pure LYZ component

**Phase separation was considered to be present for p-value < 0.10

Table 5. Comparison of the normalized aggregation rate constants, r_{RMS} values and p-values for BLG formulations to distinguish phase separation.

Formulation	Normalized Rate Constant*	r_{RMS} (Std. Error)	p-value**	Phase Separation Detected***
Pure BLG	1	0.047 (0.009)	-	-
1:1 BLG:SUC	0.05	0.060 (0.014)	0.123	NO
1:1 BLG:TRE	0.05	0.074 (0.007)	0.010	YES
1:1 BLG:DEX40K	0.8	0.102 (0.031)	0.045	YES
1:3 BLG:DEX40K	1.2	0.117 (0.003)	0.043	YES

* Normalized to the aggregation rate constant of pure BLG.

**p-value evaluated from the difference between the r_{RMS} BLG in the mixture and the r_{RMS} of the pure BLG component

***Phase separation was considered to be present for p-value < 0.10

8. Figures

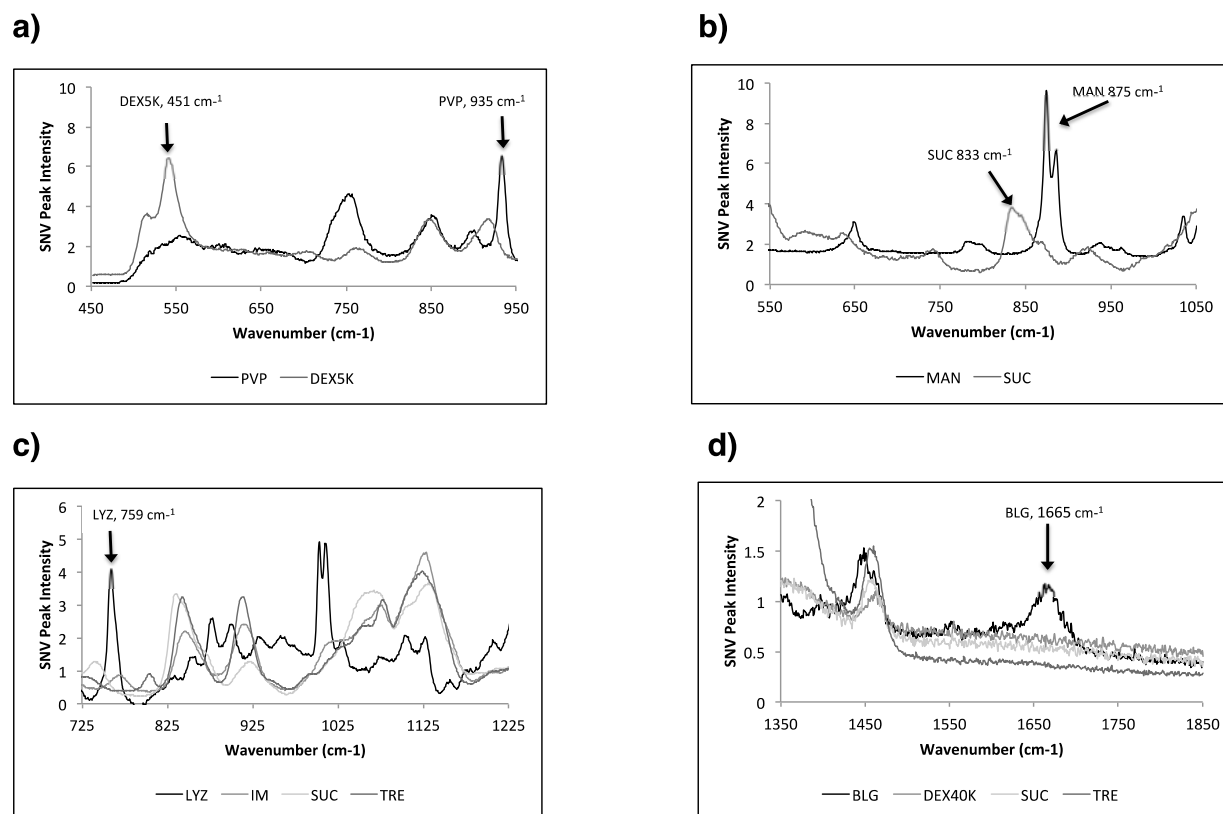


Figure 1: Analytical peaks from Raman spectra for pure components of a) PVP and DEX5K b) MAN and SUC c) LYZ in TRE, SUC or IM and d) BLG in SUC, TRE or DEX40K to identify non-overlapping peaks for analysis of lyophilized mixtures.

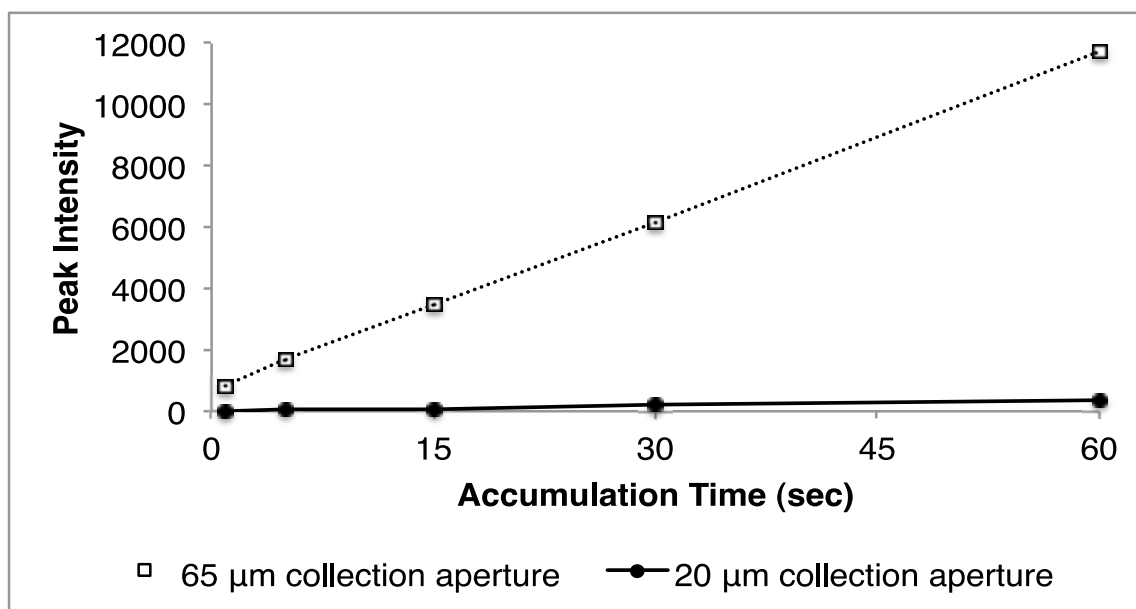


Figure 2: Raman peak intensity of LYZ at 759 cm^{-1} in 1:1 (w:w) LYZ:TRE formulations as a function of spectral accumulation time using a large collection aperture (65 μm slit) and a small collection aperture (20 μm slit). Peak intensities are the preprocessed values before application of the SNV algorithm.

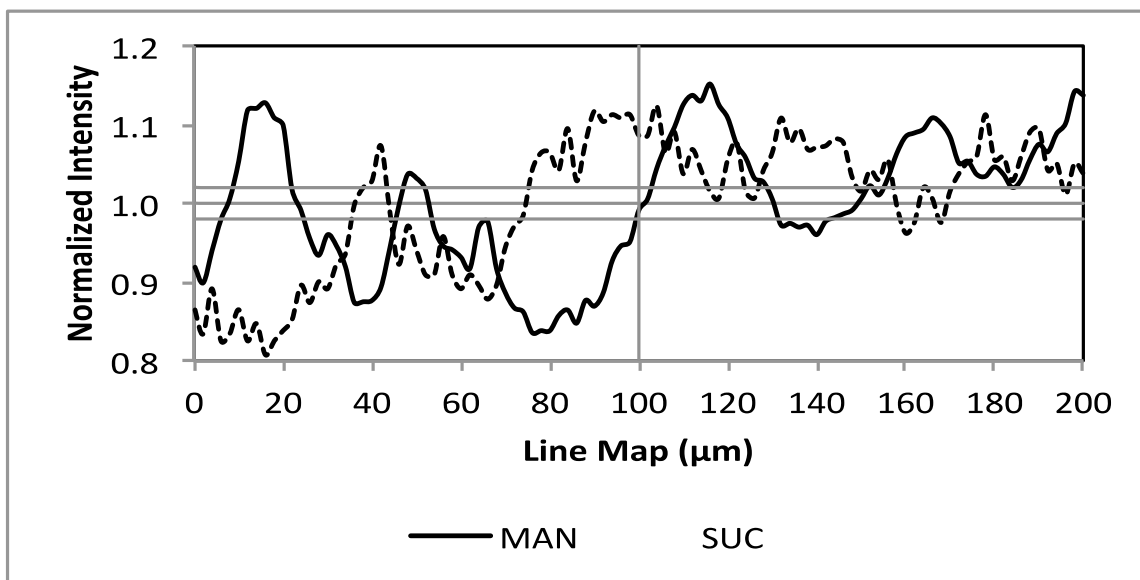
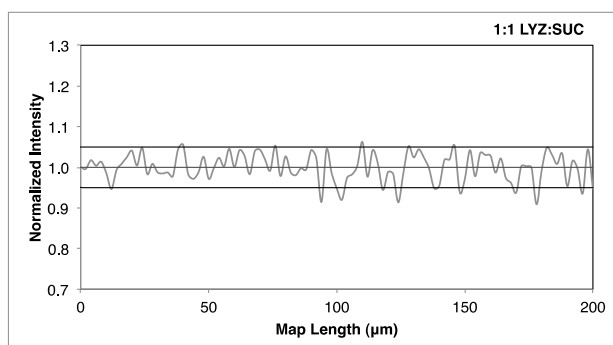
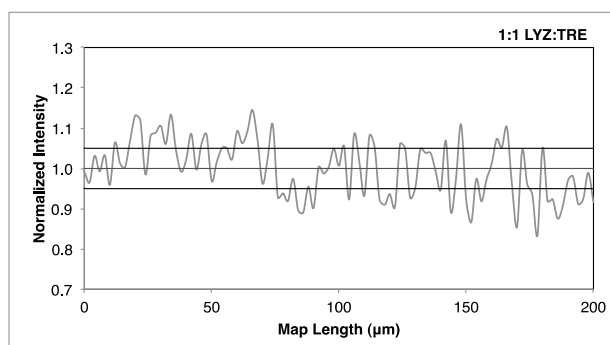


Figure 3: Line map of Raman intensities for MAN (875 cm^{-1}) and SUC (833 cm^{-1}) in a 1:2 (w:w) MAN:SUC formulation. Intensities were normalized to the average peak intensity, $\langle I(x) \rangle$, of each pure component averaged over the 200 μm line map. Horizontal lines above and below the average normalized peak intensity (1.0) represent one standard deviation of the normalized peak intensities of the pure components over a 200 μm line map.

a)



b)



c)

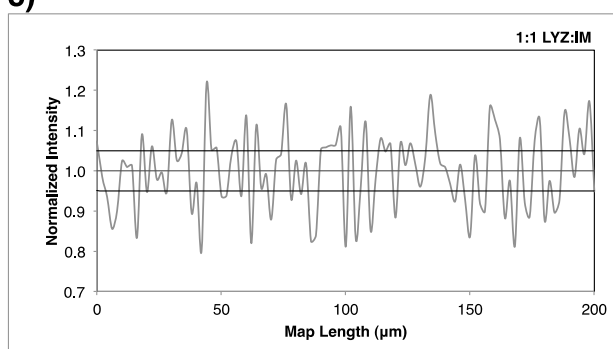


Figure 4: Line maps of Raman intensity for LYZ (759 cm^{-1}) in a) 1:1 (w:w) LYZ:SUC, b) 1:1 (w:w) LYZ:TRE and c) 1:1 (w:w) LYZ:IM formulations. Intensities were normalized to the average peak intensity, $\langle I(x) \rangle$ of pure LYZ averaged over the 200 μm line map. Horizontal lines above and below the average normalized peak intensity (1.0) represent one standard deviation of the normalized peak intensity of pure LYZ over a 200 μm line map.

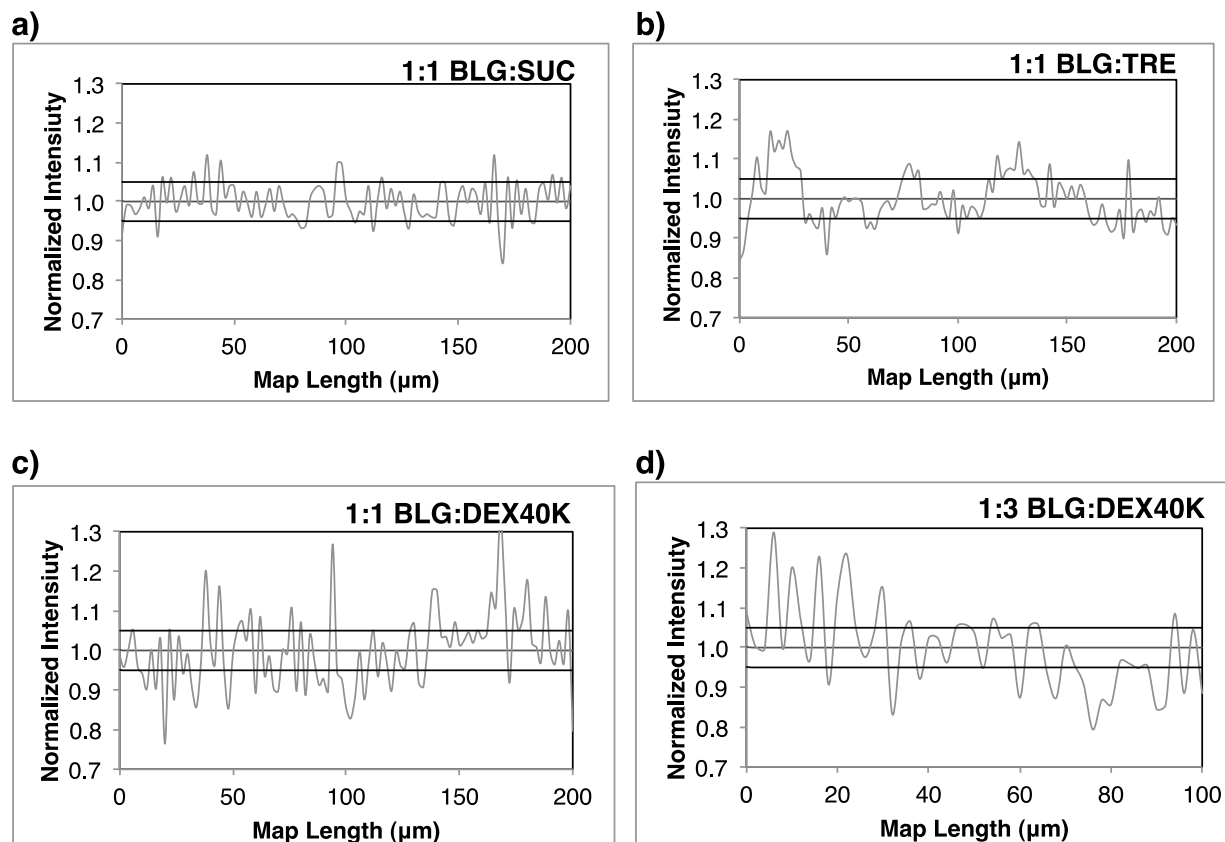


Figure 5: Line maps of Raman intensity for BLG (1665 cm⁻¹) in a) 1:1 (w:w) BLG:SUC, b) 1:1 (w:w) BLG:TRE, c) 1:1(w:w) BLG:DEX40K and d) 1:3 (w:w) BLG:DEX40K formulations. Intensities were normalized to the average peak intensity, $\langle I(x) \rangle$, of pure BLG averaged over the 200 μm line map. Horizontal lines above and below the average normalized peak intensity (1.0) represent one standard deviation of the normalized peak intensity of pure BLG over a 200 μm line map.

Chapter 7

Summary and Significance

Summary

Adequate storage stability of lyophilized proteins can be a challenge due to the high susceptibility of macromolecules to chemical and physical degradation. This instability results in lyophilized therapeutic formulations having an inadequate shelf-life and requiring refrigeration to maintain product quality. To improve long-term stability, many pharmaceutical proteins are formulated with inactive ingredients (known as excipients) to protect the protein during processing, distribution and storage. However, the mechanisms of stabilization by excipients are still not fully understood, as a result the current method of formulating is frequently done by trial and error.

The goal of this research was to better understand critical factors in stabilization of proteins in the glassy solid-state, such that long-term, ambient temperature storage can be achieved. There were two main focus of this research:

- 1) Investigate amino acids as stabilizing excipients to potentially extend the shelf-life stability of lyophilized protein formulations.
- 2) Refine a method for early detection of amorphous phase separation to better assess potential long-term stabilization issues.

To achieve the first object, the stability of sucrose-based lyophilized proteins formulated with a series of 15 amino acid additives was assessed. Properties related to the additives' chemical nature, charge, and size were evaluated. Stabilities of recombinant

human serum albumin and α -chymotrypsin against aggregation were improved by the addition of all but two of the studied amino acids. Stability improvements were attributed to the molar volume of the amino acids, indicating potential suppression of local motions within the glassy state. The study demonstrated the stabilizing effect of low levels of amino acid additives on the long-term storage stability of model freeze-dried sucrose-based protein formulations. It was made clear that stabilization by small molecule additives is not restricted to just polyols (i.e., glycerol, sorbitol) but rather can be obtained from most amino acids.

Next, the long-term storage stability of recombinant human serum albumin lyophilized with sucrose and 13 small molecule additives (i.e., amino acids, sorbitol) over a large concentration range was investigated. The optimal stabilizing amount of each additive was evaluated along with the possible relationship between the extent of stabilization and the additive's chemical and physical properties. Improvement in stability was achieved for all amino acids and sorbitol over the concentration ranges studied, with some additives having an optimal stabilizing concentration and stability improvement by a factor of five. The maximum stabilization by the amino acids correlated to the molar volume of the amino acid, further revealing the potential of the additive suppressing local motions. This study demonstrated that the stabilizing effect of the additives (amino acids and sorbitol) on the storage stability of lyophilized sucrose-based protein formulations is not necessarily restricted to a narrow range of additive concentrations.

Finally, various measures of mobility and free volume were investigated in sucrose-

based recombinant human serum albumin formulations lyophilized with amino acids (serine, methionine) and sorbitol. The goal was to apply these results to better understand the impact of small molecule additives on long-term storage stability and to assess potential mechanism(s) of stabilization. The stability of lyophilized sucrose-based formulations was vastly improved by the addition of the amino acids over a range of concentrations, however, there was no correlation between global motions and stability. In some cases, a decrease in local motions was accompanied by improved stability, but no direct correlation was found between suppressed fast dynamics and stabilization. Stabilization by the amino acids was not well correlated with a decrease in the system free volume as measured by volume changes on mixing, apparent specific volume or PALS data. While no clear predictive measures were identified, amino acids in general all showed a notable ability to improve storage stability of dried proteins. In general, amino acids can be used to further improve the long-term stability of lyophilized protein formulations, but it was not possible to assign a single cause for this effect.

Molecular mobility is hypothesized to be a major factor in affecting the physical and chemical stability of amorphous formulations; however, not all measures of mobility correlate with stability. The lack of correlation could be attributed to use of an already stable protein formulation, where the overall improvement in stability could be difficult to assess in the time frame available.

The second objective was to optimize a confocal Raman microscopic technique to more efficiently detect amorphous-amorphous phase separation in lyophilized protein

formulations. By assessing various instrument settings and sample preparation techniques, a previous method was refined to significantly reduce experimental time. The newly optimized method successfully detected amorphous-amorphous phase separation in systems where amorphous-amorphous phase separation was previously suspected but never identified. Raman microscopy has the potential to be used as an early indicator of suboptimal stability as a result of phase separation without the need for long-term stability studies.

Significance

The small molecule additives have the potential for significantly improving stability for disaccharide-based protein formulations, facilitating development of room temperature stable products. The study suggests amino acids are good candidates for the additional stabilization needed to achieve room temperature stability of protein therapeutics.

The optimized Raman method promises to be a useful tool for detection of phase separation in amorphous therapeutic formulations. Ideally, this method may be used as an early indicator of suboptimal stability due to phase separation without the need for long-term stability studies.

Greater stability of therapeutic proteins will facilitate their distribution and use globally by military troops in the battlefield and by patients in less-developed countries, where product quality is often compromised by the lack of a cold-chain and proper storage

conditions. The establishment of amino acids as stabilizers for solid-state protein formulations and the identification of an early indicator for potentially poor stability in these formulations contribute to the long-term goal of achieving more stable therapeutic proteins without the need for refrigeration.