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Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: I. Screening of 15 Amino Acids in Two Model Proteins

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ABSTRACT

In small amounts, the low molecular weight excipients—sorbitol and glycerol—have been shown to stabilize lyophilized sucrose-based protein formulations. The purpose of this study was to explore the use of amino acids as low molecular weight excipients to similarly enhance stability. Model proteins, recombinant human serum albumin and α-chymotrypsin, were formulated with sucrose in combination with one of 15 amino acid additives. Each formulation was lyophilized at 1:1:0.3 (w/w) protein–sucrose–amino acid. Percent total soluble aggregate was measured by size-exclusion chromatography before and after storage at 50°C for 2 months. Classical thought might suggest that the addition of the amino acids to the sucrose-protein formulations would be destabilizing because of a decrease in the system's glass transition temperature. However, significant improvement in storage stability was observed for almost all formulations at the ratio of amino acid used. Weak correlations were found between the extent of stabilization and both amino acid molar volume and side-chain charge. The addition of amino acids at a modest level generally improves storage stability, often by more than a 50% increase, for lyophilized sucrose-based protein formulations.

Introduction

Because of the susceptibility of proteins to both chemical and physical degradation, achieving long-term storage stability can be challenging. Lyophilization is used to stabilize liquid protein formulations via conversion of the product into a solid, providing decreased molecular motion relative to the aqueous solution as well as removing water that can be a reactant.[1] Drying to an optimal residual moisture slows, but does not completely arrest protein degradation; processes including aggregation, denaturation, deamination, and oxidation can and do still occur.[2] Excipients such as sugars and polymers are added to lyophilized formulations to protect the protein from degradation in order to achieve adequate storage stability, most often requiring refrigeration.[3-6] However, further stabilization is still required to achieve ambient temperature storage and for distribution of therapeutic protein products to warmer climates without cold chain.

Recent studies have shown that the addition of low levels of the polyols, glycerol and sorbitol, to disaccharide-based protein formulations correlated well with increased storage stability.[7-9] Normally, small molecules such as glycerol and sorbitol, that have low glass transition temperatures, are avoided in solidstate formulations as they are characteristically expected to destabilize the protein by lowering the glass transition temperatures of the matrix and increase mobility.[7,9] Although the small molecule additives do plasticize global mobility, they have been reported to antiplasticize the fast dynamics (i.e., decrease noncooperative and local motions), which has resulted in improved storage stability.[7,10] Thus, the addition of other types of low molecular additives, similar to glycerol and sorbitol, may also further enhance storage stability of proteins in the solid state. However, it is not well understood what physicochemical properties of the additives are critical to this mechanism of 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, eliminating the need for refrigeration.

Amino acids were evaluated as potential stabilizing additives, because of their broad range of available sizes and physicochemical properties. Commonly, amino acids are used in liquid protein formulations as excipients to increase solubility, buffer the system pH,

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stabilize the native protein structure, and improve stability of the protein formulation.[11] Only a few studies have investigated amino acids as stabilizing excipients in solid-state protein formulations.[12-15] For example, histidine was found to improve the stability of the enzyme lactate dehydrogenase when added to freeze-dried formulations at concentrations higher than required for buffering.[14] In a separate study, lyophilized recombinant FVIII exhibited improved storage stability when arginine, isoleucine, and glutamic acid were included in the formulation.[12] Elucidation of the properties of the small molecule additives (e.g., size, side-chain charge, polarity) that best correlate to improved storage stability will help identify the most effective small molecule stabilizers, allowing for distribution of the therapeutic product to extreme climate zones.[16]

The objective of this study was to determine whether the protein stabilization by glycerol and sorbitol could be extended to other low molecular weight excipients, and if so, to identify the properties providing stabilization. A series of 15 amino acids spanning a range of size (molecular weight, molar volume) and side change properties (charge, hydrophobicity) were screened for their stabilizing effect on two model proteins. A single concentration (13%, w/w, of total solids) was employed to allow screening of the large number of amino acids for further stabilization of 2 sucrose-based protein formulations that are considered pharmaceutically stable when stored at 4°C-8°C.[17] Sorbitol (13%, w/w, of total solids) was also used in this study for comparison to previous literature results.

Materials and Methods

Materials

Recombinant human serum albumin (rHSA) [molecular weight (MW) = 66.4 kDa, isoelectric point (p*I*) = 4.7] was purchased from Albumin Biosciences (Huntsville, AK) in solution form. The material was ultra-pure grade (purity >99%). α-Chymotrypsin (ACT) (MW = 25.6 kDa, p*I* = 8.7) was purchased from MP Biomedicals (Solon, OH) as lyophilized powder, essentially salt free with purity greater than 94%. The phosphate buffer salts, sucrose, sorbitol, and all amino acids (Table 1) were purchased from Sigma (St. Louis, MO). All excipients were of the highest grade available. All amino acids were salt free.

Protein Formulations

Before freeze-drying, rHSA and ACT were dialyzed against 2 mM sodium phosphate buffer at pH 7 using a dialysis membrane with molecular weight cut-off of 6-8000 kDa (Spectrum Laboratories, Dominguez, CA). After dialysis, final protein concentrations (by UV absorbance at 280 nm; Cary 50 Bio, Varian) of rHSA and ACT were 5 and 10 mg/mL, respectively.

All excipients were used without further purification. Bulk solutions of sucrose (100 mg/mL), sorbitol (50 mg/mL), and each amino acid (50 mg/mL) were prepared in 2 mM phosphate buffer at pH 7. Final solutions consisting of 1:1:0.3 (w/w) protein-sucrose-amino acid were prepared from the individual protein, sucrose, and amino acid bulk solutions. The total solid content of the rHSA–sucrose–amino acid and ACT–sucrose–amino acid formulations were 11.5 and 23 mg, respectively. The pH of each formulation was adjusted to pH 7 as needed using either 1 M NaOH or 1 M HCl. The amount of salt formed as a result of titrating to pH 7 was four orders of magnitude lower than the amino acid in solution; therefore, any salt formation should essentially have no effect on the stability results. Before freeze-drying, final solutions were filtered through 0.22 μm filters, and aliquots (1 mL) of the final solutions were filled into 5 cc glass tubing vials and semistoppered with low moisture-release stoppers (Daikyo Flurotech Stoppers; West Pharmaceutical, Lionville, PA).

Lyophilization

Solutions were lyophilized in a laboratory-scale freeze dryer (Durastop FTS; SP Industries, Stone Ridge, NY). Thermocouples were placed in the bottom center of at least four vials per batch. After thermal equilibration at −5°C, the shelf was cooled to −42°C at a rate of 1°C/min and held for 120 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 h. The product temperature during primary drying was maintained well below collapse temperatures. Four hours after all product thermocouples reached −32°C, the shelf was increased to 40°C at 0.1°C/min and held for 6 h. Upon completion, the shelf was lowered to 5°C, and the vials were stoppered under nitrogen (650-700 Torr) and crimped with aluminum seals. All freeze-dried samples showed no visible

Table 1
Physical and Chemical Characteristics of the 15 Amino Acids Used in the 1:1:0.3 Protein-Sucrose-Amino Acid Formulations[18,22]

Side-Chain Classification (Charge/Polarity)	Additive	Molecular Weight (g/mol)	Density (g/mL)	Molar Volume (mL/mol)	p*I*	Dipole Moment (D)	Hydrophobicity	Side-Chain Flexibility	Potential Side-Chain Hydrogen Bonds
Hydrophobic	Alanine	89.1	1.37[a]	65.0	6.1	0	0.806	Low	0
	Valine	117.2	1.27	92.3	6.0	0.06	0.923	Low	0
	Leucine	131.2	1.20	109.3	6.0	0.09	0.918	Moderate	0
	Isoleucine	131.2	1.17	112.1	6.0	0.07	1.000	Moderate	0
	Methionine	149.2	1.30[a]	114.8	5.7	1.80	0.811	High	0
	Phenylalanine	165.2	1.32	125.2	5.9	0.29	0.951	Moderate	0
Polar	Serine	105.1	1.55[a]	67.8	5.7	1.83	0.601	Low	3
	Threonine	119.1	1.46[a]	81.6	5.6	1.79	0.634	Low	3
	Glutamine	146.1	1.32	110.7	5.7	3.89	0.430	High	5
	Citrulline	175.2	1.29	135.8	5.9	–	–	–	–
Positive	Lysine	146.2	1.33[a]	109.9	9.5	9.07	0.263	High	3
	Histidine	155.2	1.41	110.1	7.6	4.04	0.548	Moderate	3
	Arginine	174.2	1.33	131.0	10.8	5.78	0.000	High	7
Negative	Aspartic acid	133.1	1.64[a]	81.2	3.0	4.33	0.417	Moderate	4
	Glutamic acid	147.1	1.57	93.7	3.1	6.13	0.458	High	4
–	Glycerol[b]	92.1	1.26	73.1	–	–	–	–	–
	Sorbitol[b]	182.2	1.49	122.3	–	–	–	–	–

[a] Amino acid crystalline density values measured directly using He pycnometer. All other values are from literature.
[b] The molecular weight, density, and molar volume of glycerol and sorbitol are included for comparison purposes. The amino acid properties (crystalline density, p*I*, dipole moment, hydrophobicity, size chain flexibility, and potential side-chain hydrogen bonds) were obtained from literature and the National Center for Biotechnology Information online database.