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Process and Formulation Effects on Protein Structure in Lyophilized Solids Using Mass Spectrometric Methods

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ABSTRACT

Myoglobin (Mb) was lyophilized in the absence (Mb-A) and presence (Mb-B) of sucrose in a pilot-scale lyophilizer with or without controlled ice nucleation. Cake morphology was characterized using scanning electron microscopy, and changes in protein structure were monitored using solid-state Fourier-transform infrared spectroscopy, solid-state hydrogen-deuterium exchange–mass spectrometry, and solid-state photolytic labeling–mass spectrometry (ssPL-MS). The results showed greater variability in nucleation temperature and irregular cake structure for formulations lyophilized without controlled nucleation. Controlled nucleation resulted in nucleation at ~(−5°C) and uniform cake structure. Formulations containing sucrose showed better retention of protein structure by all measures than formulations without sucrose. Samples lyophilized with and without controlled nucleation were similar by most measures of protein structure. However, ssPL-MS showed the greatest photoleucine incorporation and more labeled regions for Mb-B lyophilized with controlled nucleation. The data support the use of solid-state hydrogen-deuterium exchange–mass spectrometry and ssPL-MS to study formulation and process-induced conformational changes in lyophilized proteins.

Introduction

Proteins are often marketed in lyophilized form or stored as lyophilized powders after purification and before formulation. Lyophilization begins with freezing, followed by primary drying to remove bulk ice by sublimation and secondary drying to desorb unfrozen water. Proteins are subjected to various stresses during lyophilization, including freeze concentration and denaturation at the ice surface, pH shifts, and dehydration-induced aggregation.[1-4] Proteins can be protected from some of these stresses by modifying the formulation and using stabilizing excipients.[5-8] However, the process itself can play a role in determining critical quality attributes of the product. For example, the thermal history of freezing may result in the formation of mannitol hydrate in mannitol-containing formulations, which seem to be metastable and can release water during storage, adversely influencing the long-term storage stability of the drug product.[9-11] Inadequate drying temperature or time can also result in product failure on account of increased moisture content, and processing or storage above the glass transition temperature (T_g) can result in degradation.[12,13] Cake elegance can be adversely affected by aggressive processing above the collapse temperature, producing various degrees of macrocollapse and microcollapse.[14,15] Higher temperatures during lyophilization can degrade reducing carbohydrate excipients via the Maillard reaction,[16] which may reduce their stabilizing effects.

The freezing step is critical because parameters such as the degree of supercooling and rate of freezing can affect the morphology of ice crystals, which in turn affects the rate of primary drying.[17,18] When a solution is supercooled to a large degree, ice nucleation occurs at lower temperatures with little time for ice crystal growth, resulting in smaller pores in the dried solid. These small pores offer greater resistance to the flow of water vapor through the porous bed of partially dried solids. This necessitates the use of a longer primary drying step to remove crystalline water. In contrast, a lower degree of supercooling is associated with a

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slower rate of freezing and a relatively small number of large ice crystals. Because ice nucleation is stochastic, cycles without controlled freezing are expected to nucleate over a range of temperatures, resulting in longer freezing times and heterogeneous ice crystal morphology. Variability is also introduced by vial position within the lyophilizer chamber[19] because vials near the door and walls of the lyophilizer chamber receive more heat via radiation than those near the center of the chamber. Together, these factors result in intervial and interbatch heterogeneity. If left uncontrolled, this variability may be magnified when a process is scaled from a laboratory bench-top lyophilizer to a production freeze dryer. Heat- and mass-transfer differences between pilot and production freeze dryers may also play a role, so that the same lyophilization cycle may produce variable product critical quality attributes at different scales. Controlling the freezing step is critical to producing uniform ice crystal morphology, resulting in less variability between samples and faster drying. The freezing rate can also affect product stability because smaller ice crystals formed by fast freezing present a greater surface area for potential protein adsorption and unfolding. Aggregation at the protein solution-ice interface has been implicated in the lyophilization-induced instability of human growth hormone,[20] recombinant human factor XIII,[21] lactate dehydrogenase, and immunoglobulin G.[3]

Strategies to control nucleation include the use of an ice fog as a seeding technique and rapid depressurization to induce spontaneous nucleation. Although the effect of controlled ice nucleation on primary drying time has been well documented,[22-25] its effect on protein structure is not well understood. Controlled nucleation at a lower degree of supercooling would be expected to result in relatively larger ice crystals with lower surface area for protein adsorption. This would be expected to produce a product that is more stable than one lyophilized without controlled nucleation. The effect of depressurization-induced controlled nucleation on product characteristics has been reported for a monoclonal antibody.[26] Although the drying time was reduced by ~10 h and cake appearance improved to some extent with controlled nucleation, there was no significant impact on aggregation as detected by UV spectroscopy and size-exclusion chromatography. Secondary structure was not altered significantly, as quantified by circular dichroism spectroscopy. The process did not affect binding to protein-A, suggesting that the tertiary structure was also intact, at least at the binding site. Other studies investigated the effect of different lyophilization cycles on protein conformation and cake structure.[27,28] Although cycle variations typically led to altered cake morphology as detected using scanning electron microscopy (SEM), conformational changes could not be detected using conventional solid-state Fourier-transform infrared spectroscopy (ssFTIR) and solution-state circular dichroism and fluorescence spectroscopy.

In this work, the effects of controlled nucleation and lyophilizer scale on protein structure were examined using high-resolution mass spectrometric methods: solid-state hydrogen-deuterium exchange–mass spectrometry (ssHDX-MS) and solid-state photolytic labeling–mass spectrometry (ssPL-MS). To our knowledge, this is the first application of these methods to both process and formulation effects. The ControLyo® depressurization technology was used for controlled nucleation. Myoglobin (Mb) lyophilized in a LyoStar freeze dryer with or without controlled nucleation showed no significant changes in structure at the backbone and side-chain levels, as determined by ssFTIR, ssHDX-MS, and ssPL-MS, respectively. However, formulation effects were dominant, and protein structure was better protected at the backbone in the presence of sucrose. The results indicate that, at least for the model protein used here, the local structure remains unaltered by controlled nucleation and that ssHDX-MS and ssPL-MS can be used to detect process- and formulation-induced changes in protein structure.

Materials and Methods

Materials

Equine skeletal muscle holomyoglobin, sucrose, potassium phosphate dibasic, and ammonium bicarbonate were purchased from Sigma Aldrich (St. Louis, MO). Potassium phosphate monobasic (anhydrous) was purchased from Amresco (Solon, OH). D_2O was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA), and photo-leucine (L-2-amino-4, 4′-azipentanoic acid) from Thermo Scientific (Rockford, IL). Mass spectrometry-grade water, acetonitrile, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Spectra/Por dialysis tubing (MWCO 8000-10000 Da) was used to dialyze the protein before formulation (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Syringes (Beckton, Dickinson and Company, Franklin Lakes, NJ) and 0.2-μm Acrodisc® syringe filters (Pall Corporation, Ann Arbor, MI) were used to filter the dialysate.

Sample Preparation

Mb was dissolved in potassium phosphate buffer (2.5 mM, pH 7.4) and dialyzed overnight against the same buffer using dialysis tubing. The dialyzed protein was filtered using a syringe filter and its concentration measured using UV spectroscopy (8453 UV-Vis, Agilent Technologies, Santa Clara, CA) and the molar extinction coefficient ε_{555} nm = 12.92 $mM^{-1}cm^{-1}$ (obtained from Sigma Aldrich product information sheet for equine skeletal muscle myoglobin, product M0630). This stock solution (345 μM) was used for all formulations. A 20-mg/mL stock solution of sucrose was prepared by dissolving sucrose in potassium phosphate buffer (2.5 mM, pH 7.4) and stored at 4°C until use. Similarly, a 30.9-mM stock solution of photoleucine (pLeu) was prepared using the same buffer and stored at 4°C until use.

Two formulations were prepared for lyophilization: a control formulation containing Mb and buffer (“Mb-A”) and a formulation containing Mb, sucrose, and buffer (“Mb-B”). Stock solutions of Mb, sucrose, and buffer were mixed such that the final Mb concentration was 70 μM and the ratio of Mb to sucrose was 1:1 w/w. For photolytic labeling studies, pLeu was added to Mb-A and Mb-B such that the molar ratio of pLeu to Mb was 100:1. The weight fractions of each component are listed in Table 1. The formulations were filled in glass tubing vials (USP type I glass; 2 mL capacity) with 13-mm necks. The fill volume was 500 μL for ssHDX-MS, ssPL-MS, thermogravimetric analysis, X-ray diffraction, and solid-state Fourier-transform infrared spectroscopy samples. A 3-mL fill in 10-mL-capacity glass beakers was used for SEM.

Lyophilization

Both Mb-A and Mb-B formulations were lyophilized with and without controlled nucleation during the freezing step. For freezing with controlled nucleation (LyoStar 3 with ControLyo®, SP Industries, Inc., Gardiner, NY), the vials were equilibrated at 5°C for

Table 1
Weight Fractions of Components of Lyophilized Formulations

Lyophilized Formulation	% w/w			
	Mb	Sucrose	Buffer	pLeu
Mb-A	91.7	N/A	8.3	N/A
Mb-B	42.9	42.9	14.1	N/A
Mb-A + pLeu	46.0	N/A	15.1	38.8
Mb-B + pLeu	31.5	31.5	10.4	26.6

Mb, myoglobin; pLeu, photoleucine (L-2-amino-4,4′-azipentanoic acid).