Protein Quantity on the Air–Solid Interface Determines Degradation Rates of Human Growth Hormone in Lyophilized Samples

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Received 22 January 2014; revised 11 February 2014; accepted 17 February 2014

Published online 12 March 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23926

ABSTRACT: Recombinant human growth hormone (rhGH) was lyophilized with various glass-forming stabilizers, employing cycles that incorporated various freezing and annealing procedures to manipulate glass formation kinetics, associated relaxation processes, and glass-specific surface areas (SSAs). The secondary structure in the cake was monitored by infrared and in reconstituted samples by circular dichroism. The rhGH concentrations on the surface of lyophilized powders were determined from electron spectroscopy for chemical analysis. Glass transition temperature (T_g), SSAs, and water contents were determined immediately after lyophilization. Lyophilized samples were incubated at 323 K for 16 weeks, and the resulting extents of rhGH aggregation, oxidation, and deamidation were determined after rehydration. Water contents and T_g were independent of lyophilization process parameters. Compared with samples lyophilized after rapid freezing, rhGH in samples that had been annealed in frozen solids prior to drying, or annealed in glassy solids after secondary drying retained more native-like protein secondary structure, had a smaller fraction of the protein on the surface of the cake, and exhibited lower levels of degradation during incubation. A simple kinetic model suggested that the differences in the extent of rhGH degradation during storage in the dried state between different formulations and processing methods could largely be ascribed to the associated levels of rhGH at the solid–air interface after lyophilization. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1356–1366, 2014

Keywords: protein structure; protein formulation; lyophilization; stability; mobility; specific surface area; annealing; surface degradation; growth hormone

INTRODUCTION

Lyophilization is widely accepted as an effective method to improve long-term stability of pharmaceuticals, especially therapeutic protein products.[1] In glassy lyophilized solids, both physical and chemical degradation processes are greatly hindered.[1] However, storage of proteins in glassy solid formulations does not always guarantee a desired shelf life.[2,3] For decades, efforts have been made to choose appropriate formulations and design robust lyophilization cycles to yield stable protein products.[1] It has been widely documented that disaccharides (sucrose and trehalose) are effective stabilizers during lyophilization and storage, and this stabilizing effect has been ascribed to thermodynamic and/or kinetic stabilization mechanisms.[4–6] In addition, the lyophilization cycle applied to a given formulation may determine not only the morphology of the cake and physical properties of the glass, but also the stability of the protein during storage in the dried formulation.[1,4,6–9] For example, the stability of methionyl human growth hormone in dried solid formulations was shown to depend not only on the type of stabilizer included in the formulation, but also on the drying method that was used.[9] Despite some insights into mechanisms by which excipients provide stability to proteins, developing a formulation that provides adequate protection against protein damage is still a semiempirical exercise, as is the design of a lyophilization cycle that provides optimal protein stability for a given formulation.

A conventional lyophilization cycle consists of freezing, primary drying, and secondary drying steps. The freezing step is of paramount importance.[10,11] During freezing, most of the water present in the original liquid (about 80%[10]) is crystallized into essentially pure ice, which results in a freeze-concentrated solution for the remaining formulation. The pH of the freeze-concentrated liquid may undergo a shift because of the preferential precipitation of buffer components, potentially contributing to protein denaturation.[12,13] In addition, the rates of ice nucleation and crystal growth have large impacts on the ice morphology and ice–liquid interfacial area, and consequently on the final solid products' specific solid–air interfacial area formed after drying.[7,10,14,15]

Several strategies[10,16] have been developed to manipulate the initial stages of the lyophilization cycle, such as shelf-ramp

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Journal of Pharmaceutical Sciences, Vol. 103, 1356–1366 (2014)

cooling, annealing, controlled ice nucleation, and fast freezing by liquid N_2. Shelf-ramp cooling is a standard cooling method in commercial freeze-drying, during which shelf temperature of the lyophilizer is decreased in a roughly linear fashion. In this case, the maximum cooling rate is limited by the cooling capacity of lyophilizer.[17,18] Shelf-ramp cooling typically results in a high degree of supercooling prior to the initiation of freezing, followed by a period characterized by rapid nucleation of a large number of ice crystals.[14] Consequently, many small ice crystals are formed. During the primary drying portion of the lyophilization process, ice crystals are removed by sublimation, and the interface between the glass and the voids left behind contribute the specific surface area (SSA) of the resulting glassy solid.[15,19,20] The large number of relatively small ice crystals formed after shelf-ramp freezing in turn yields lyophilized cakes with large surface areas.[10]

Annealing refers to an additional step that may be added after freezing, during which the sample temperature is maintained between the ice melting temperature and the glass transition temperature of the maximally freeze-concentrated solution, T_g'[11,19] (or the eutectic melting temperature of crystalline excipients, if that temperature is greater than T_g'). Because of the both enhanced mobility of water and the contributions of surface energy to the elevated chemical potential of water in smaller crystals, water is transported from small ice crystals and redeposits onto large ice crystals.[10] As a result of this Ostwald ripening, larger ice crystals are generated during annealing, which in turn results in lyophilized cakes with reduced SSAs.[11] For the purposes of this report, this type of annealing process will be termed "predrying annealing."

We note that Ostwald ripening also occurs in lyophilization cycles that use shelf-ramp cooling without an annealing step, but to a lesser degree, because the length of time available for ripening (the time during which ice crystals are present and the temperature is above T_g') is much shorter in these cycles. To reduce the extent of Ostwald ripening even further, fast freezing methods may be used to limit the time that samples spend at temperatures between the ice melting temperature and T_g'. One method of fast freezing is to immerse samples contained in vials into liquid N_2 (N_2 immersion). Fast freezing also may be achieved by spraying liquid droplets of sample directly into liquid N_2 (N_2-droplet freezing).[13,21–24] In the N_2 immersion procedure, the relatively low thermal conductivity of the glass vials limits heat transfer, making the effective cooling rate slower than that which can be achieved using the N_2-droplet-freezing method.[10] Compared with the standard shelf-ramp cooling, both of these two fast freezing methods result in smaller ice crystals and larger glassy matrix-SSAs.[10]

Another type of annealing (herein termed "postdrying annealing") may be implemented by briefly incubating dry, glassy samples at a high (but sub-T_g) temperature at the end of the secondary drying step of the lyophilization cycle. Wang et al.[25] reported that postdrying annealing could enhance protein stability. The stability increase was presumably a result of relaxation processes that lead to slower motions in the glassy state.[25]

The current study examined formulations of recombinant human growth hormone (rhGH) in the presence of three glass-forming excipients: sucrose, trehalose, and hydroxyethyl starch (HES). These formulations were lyophilized using five different methods, which yielded glassy solids with different SSAs, surface protein contents, glassy state mobilities, and degrees of retention of native secondary structure. Because we anticipate that protein molecules located on the surface of lyophilized glassy solids will have significantly faster degradation rates, we hypothesize that the extent of rhGH degradation during storage in various dried solid formulations prepared by different processing methods can largely be ascribed to the resulting levels of rhGH found at the solid-air interface after lyophilization.

MATERIALS AND METHODS

Materials

Recombinant human growth hormone was expressed in *Escherichia coli* and purified as described previously.[8,26] HES (Viastarch) was purchased from Fresenius (Graz, Austria), and sucrose and trehalose were purchased from Mallinckrodt Baker (Phillipsburg, New Jersey). All other chemicals were purchased as reagent grade or higher. Lyophilization glass vials (5 mL, product number 68000318) and butyl rubber stoppers (product number 19560042) were purchased from West Pharmaceutical Services (Linville, Pennsylvania).

Methods

Formulation and Lyophilization Cycle Design

Recombinant human growth hormone was formulated at a concentration of 1 mg/mL in one of the three formulations. In addition to rhGH, each formulation contained 2 mM sodium phosphate at a pH of 7.4, as well as 5% (w/v) of HES, trehalose, or sucrose. Lyophilization was performed using a FTS Lyostar I system. An aliquot (1 mL) of each rhGH formulation was pipetted into vials, and lyophilized with one of five different lyophilization cycles, denoted as standard lyophilization, predrying annealing lyophilization, postdrying annealing lyophilization, N_2 immersion lyophilization, and N_2-droplet-freezing lyophilization.

In the standard lyophilization cycle, sample vials were loaded onto the shelf, which was at room temperature. The shelf temperature was reduced to 10°C, and samples were equilibrated at this temperature for 1 h. Shelf temperature was then decreased to −5°C at 1°C/min, kept at −5°C for 20 min, and then decreased to −45°C at 1.3°C/min. Samples were kept frozen at −45°C for 400 min. Primary drying was then initiated and performed at a shelf temperature of −20°C and a chamber pressure of 70 mTorr for 1400 min. Secondary drying was then started by increasing the shelf temperature to 33°C at a rate of 0.3°C/min. Samples were held at 33°C and 70 mTorr for 4 h. Finally, vials were sealed in the chamber under dry nitrogen.

For the predrying annealing lyophilization cycle, an additional annealing step was added to the standard cycle. After samples were kept frozen at −45°C for 400 min, shelf temperature was increased to −5°C over 30 min. Then, shelf temperature was kept at −5°C for 6 h before cooling to −45°C. The shelf temperature was kept at −45°C for 6 h, and then the primary and secondary drying steps followed the same protocol as in the standard lyophilization cycle.

Postdrying annealing was performed using the same protocol as standard lyophilization cycle, except that after the standard secondary drying step, shelf temperature was increased up to 50°C at a rate of 0.3°C/min, and held at 50°C for 6 h before ending the cycle.