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Research paper

Storage stability of keratinocyte growth factor-2 in lyophilized formulations: Effects of formulation physical properties and protein fraction at the solid–air interface

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ABSTRACT

Lyophilized formulations of keratinocyte growth factor-2 (KGF-2) were prepared with a range of disaccharide (sucrose or trehalose) and hydroxyethyl starch (HES) mass ratios. Protein degradation was assessed as a function of time of storage of the dried formulations at 40, 50 and 60 °C. Lyophilized and stored samples were rehydrated, and protein degradation was quantified by measuring loss of monomeric protein with size exclusion chromatography and by determining chemical degradation in the soluble fraction with reverse-phase chromatography. The secondary structure of the protein in the lyophilized formulations was studied with infrared spectroscopy. The magnitudes of degradation were compared the key physical properties of the formulations including retention of protein native secondary structure, glass transition temperature (Tg), inverse mean square displacements $\langle u^2 \rangle^{-1}$ for hydrogen atoms (fast β relaxation), and the relaxation time τ^{β}, which correlates with relaxation due to fast Johari–Goldstein motions in the glass (Xu et al., 2013) [1]. In addition, specific surface areas of the lyophilized formulations were determined by Brunauer–Emmet–Teller analysis of krypton adsorption isotherms and used to estimate the fraction of the KGF-2 molecules residing at the solid–air interface. KGF-2 degradation rates were highest in formulations wherein the protein's structure was most perturbed, and wherein β relaxations were fastest, but the dominant factor governing KGF-2 degradation in freeze-dried formulations was the fraction of the protein found at the glass solid–air interface.

1. Introduction

A lyophilized formulation is often the choice of dosage form when an aqueous solution formulation of a therapeutic biologic molecule is not sufficiently stable to achieve its desired shelf life. However, even in lyophilized formulations, proteins are prone to instabilities such as aggregation or chemical degradation. To minimize protein degradation, various excipients are added to the lyophilized formulation [2]. Key to the development of robust formulations that offer proteins protection against degradation is the formation of a glassy matrix by the added excipients. Commonly used glass-forming excipients include the disaccharides trehalose and sucrose, as well as polymers such as hydroxyethyl starch (HES) [2].

During storage proteins in lyophilized formulations that contain stabilizing excipients can still degrade, albeit slowly. The factors that control the rates of this degradation are not well understood, and there is still substantial debate about the physical properties of lyophilized formulations that govern rates of degradation. The extent of native structural retention during freeze-drying [3–5] and the dynamic properties of the glassy matrix [6,7] are most often considered critical to the long-term storage stability of proteins in lyophilized formulations. Other phenomena, such as phase separation [8–12] and interfacial adsorption [13,14] of protein molecules have been shown to affect protein stability during

Abbreviations: KGF-2, keratinocyte growth factor-2; HES, hydroxyethyl starch; BET, Brunauer–Emmett–Teller.

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lyophilization, but their effects on stability during storage have not been investigated. In the current study, we examine the possibility that in addition to the degree of native protein structural retention and glass dynamics properties, the accumulation of proteins on the surface of lyophilized solids may be of importance in determining degradation rates.

In the current study as a model protein, we used keratinocyte growth factor-2 (KGF-2), a protein with a beta trefoil structure (with 12 beta strands folded into three units with each unit consisting of beta–beta–beta–loop–beta) that is found in numerous growth factors and some human interleukins [15]. The storage stability of KGF-2 was examined in various glassy, lyophilized formulations that contained mixtures of HES and the disaccharides sucrose or trehalose. The rates of aggregation and chemical degradation of KGF-2 were measured, and correlated with measures of native secondary structure retention and glassy matrix dynamics. In addition, we measured the specific surface areas of various lyophilized disaccharide–HES formulations, and used these data to estimate the amount of KGF-2 present at the glass–air interface. We propose a simple model that accounts for the possibility that the protein molecules adsorbed at interfaces may degrade more rapidly than protein molecules found in the bulk glass.

2. Materials and methods

2.1. Materials

KGF-2 was a generous donation from Human Genome Sciences Inc, Rockville, Maryland. Hydroxyethyl starch/viastarch (HES) was obtained from Fresenius Kabi, Austria, GmbH. Sucrose was purchased from Pfanstiehl laboratories, Waukegan, IL, and trehalose and sodium phosphate were obtained from J.T. Baker, PA.

2.2. Preparation of KGF-2 freeze-dried formulations

KGF-2 was dialyzed into 2 mM sodium phosphate buffer, pH 6.2 and subsequently concentrated to 10 mg/ml in centrifugal concentrators (Vivaspin® 20) at 10 °C. Formulation excipients and their percent weight masses used for preparing formulations of KGF-2 are listed in Table. 1. Each of the initial aqueous solution formulations of KGF-2 was prepared by weighing excipients into a 50 ml container followed by the addition of 5 ml of the dialyzed and concentrated 10 mg/ml concentrated KGF-2 solution. 2 mM sodium phosphate buffer was added to obtain a final volume of 50 ml and a final protein concentration of 1 mg/ml. Aliquots of 1 ml solution were pipetted into 5 cc glass vials (13 mm FNT BB LYO). Vial filling took place in a cold room at approximately 2–8 °C. The vials were then partially stoppered using double vent Fluorotec rubber stoppers (Daikyo Fluorotec stoppers, West Pharmaceutical, Lititz, PA).

Table 1
Formulation excipients and their percent weight masses used for preparing formulations of KGF-2.

Formulation#	Formulation additives, % by weight	Buffer used
1	Trehalose 5%	2 mM sodium phosphate, pH 6.2
2	Trehalose 4%, HES 1%	
3	Trehalose 2.5%, HES 2.5%	
4	Trehalose 1%, HES 4%	
5	Sucrose 5%	
6	Sucrose 4%, HES 1%	
7	Sucrose 2.5%, HES 2.5%	
8	Sucrose 1%, HES 4%	
9	HES 5%	

2.3. Freeze drying conditions for KGF-2 formulations

The glass vials containing the liquid KGF-2 formulations were loaded onto the shelves of a FTS Durastop® microprocessor-controlled freeze-dryer (SP Industries, Warminster PA) equipped with a Dura-dry MP® condenser unit. The initial shelf temperature of the freeze dryer was 10 °C. Vials were allowed to equilibrate at this temperature for 60 min. The shelf temperature then was reduced to −5 °C at 1 °C/min and held for 20 min, followed by a second ramp to −45 °C at 1.3 °C/min. After 30 min at a shelf temperature of −45 °C the chamber was evacuated to a pressure of 70 mTorr, and shelf temperature was increased from −45 °C to −20 °C over ≈10 min (2.5 °C/min) and held at this temperature for the duration of primary drying (1400 min). At the end of primary drying, the shelf temperature was increased from −20 °C to +33 °C over 3 h (a ramp rate of ≈0.3 °C/min) and held at this temperature for 2 h for secondary drying. After secondary drying was completed, the chamber was vented with dry nitrogen and the vials were stoppered within the freeze-drying chamber. The vial stoppers were later crimped onto the vials with aluminum seals.

2.4. Water content determination in KGF-2 lyophilized formulations

The residual water content in the freeze-dried formulations was determined using a Mettler DL37 coulometric moisture analyzer (Hightstown, NJ) with pyridine free vessel solutions (Photovolt Instruments, Inc., St. Louis Park, MN). Water standards were used to verify the accuracy of the instrument. The vials with freeze-dried samples were placed in a glove box (purged with dry nitrogen gas), and the dried formulations were dissolved in anhydrous dimethylformamide (DMF) (<50 ppm water). Water content in the samples was determined after subtraction of water level in DMF.

2.5. Surface area measurement

Surface areas of lyophilized formulations were calculated from krypton adsorption isotherms measured using a Quantachrome Autosorb-1 (Boynton Beach, FL). For each formulation, the contents of five vials of lyophilized placebo formulation without protein were inserted into the sample cell, and then the cell with samples was placed under vacuum to remove the moisture.

Krypton adsorption at liquid nitrogen temperature was measured at five points, with helium serving as an inert carrier gas. Brunauer–Emmett–Teller (BET) adsorption theory [16] was used – over a pressure range of 0.10–0.30 of the saturation pressure of krypton – to calculate the specific surface areas.

2.6. Storage stability of KGF-2

The lyophilized KGF-2 formulations were incubated in temperature-controlled incubators at 40, 50 or 60 °C. At various time points (0, 1, 4 9 and 16 weeks), samples were removed for the analyses described below.

2.7. Infrared spectroscopic (IR) analysis of KGF-2 secondary structure

IR spectra of KGF-2 in aqueous solution and in freeze-dried formulations were acquired using a BOMEM instrument (ABB Bomem Inc., Quebec, Canada) equipped with GRAMS® software (Galactic Industries Corp., Salem, NH). The IR spectrum of native KGF-2 in aqueous solution was obtained first. An aliquot of 10 mg/ml KGF-2 in 10 mM sodium citrate buffer, pH 6.2, was placed in a variable path length cell with CaF_2 windows (Biotools Inc., Jupiter, FL). Using a path length of 6 μm, a total of 128 interferograms in single-beam transmission mode (resolution of 4 cm^{-1}) were collected