RESEARCH ARTICLE

Mechanism of Protein Stabilization by Trehalose During Freeze-Drying Analyzed by *In Situ* Micro-Raman Spectroscopy

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ABSTRACT: Raman investigations were performed *in situ* during freeze-drying of two model proteins, lysozyme and chymotrypsinogen. The structures of proteins dissolved in 0–30 wt % solutions of trehalose in D_2O were monitored with the fingerprint (800–1800 cm^{-1}) spectrum, simultaneously with freezing, ice sublimation, and water desorption analyzed in the O-D stretching (2200–2700 cm^{-1}) region. In the absence of trehalose, the main changes were detected at the end of primary drying, and correspond to distortion and disordering of secondary structures. A stabilizing effect of trehalose was evidenced in primary and secondary drying stages. Raman images were calculated after freezing and primary drying, providing the distributions of trehalose, water, and protein which occur during the first two stages of the lyophilization cycle. Raman images show a slight heterogeneity in the degree of protein denaturation at the end of primary drying, in relation with the structure of the frozen product observed during freezing. The ability of trehalose to make the protein more rigid was determined as responsible for the protein stabilization during a lyophilization cycle. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: lyophilization; Raman spectroscopy; proteins; trehalose; protein structure; stability; Raman mapping

INTRODUCTION

Freeze-drying is widely used in the pharmaceutical and biotechnology industries to improve the stability and long-term storage of protein drugs.[1] However, the protein is exposed to different kinds of stresses (low temperature, dehydration, formation of ice crystals, changes in pH, and concentration) during a freeze-drying cycle.[2] Formulations are usually empirically prepared, to avoid the protein denaturation mainly against freezing and drying stresses, without precise knowledge about the origin of the denaturation. Consequently, different kinds of stabilizing solutes (sugars, polyols, polymers, etc.) are used to act as cryoprotectant during freezing and lyoprotectant during the drying stages.

In a recent study,[3] the stability of different types of globular proteins was monitored *in situ* during a lyophilization cycle by micro-Raman spectroscopy. In the absence of bulking and bioprotective agents, spectral changes were systematically detected at the end of the first drying stage, during the removal of the residual ice. No further structural transformation was detected during the second drying stage. The spectral changes have been interpreted in terms of local disordering related to the distortion of the structural elements (α-helices and β-sheets), induced by ice sublimation around the protein surface.

It is recognized that sugars are both efficient cryoprotectant and lyoprotectant. Two main hypotheses were suggested to understand the stabilization mechanisms of proteins during freeze-drying and long-term storage. The vitrification hypothesis[4] based on the formation of a glassy matrix, and the water replacement hypothesis corresponding to the substitution of the hydration water by excipients bound to the protein via hydrogen bonds.[5] It was also reported[6] that the stability of biomolecules in the dried state could result from both effects, that is, H-bonding between sugar and biomolecules and the glass formation of the sugar–protein mixture. However, most of the investigations[7,8] have been carried out at the end

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of the freeze-drying cycle, in the dried state, or after reconstitution, to analyze structural changes of proteins in the presence or absence of excipients. Consequently, the action of sugar on protein is partially analyzed, and these investigations only provide a picture of the dried state, occulting crucial information on protein–solvent/solutes interactions, and denaturation during freezing and drying stages.

In the present study, the influence of sugar on proteins was analyzed *in situ* during the three stages of freeze-drying for different sugar concentrations, to give a better insight on the stabilization mechanisms of proteins by sugar during freeze-drying. Two model proteins characterized by different structural conformations were analyzed *in situ* during a freeze-drying cycle, in the presence or absence of sugar. Lysozyme (LYS, 16 kDa) is mainly composed of α-helices (40%–45%) and also contains a minor contribution of β-sheet structures. Chymotrypsinogen (CHYM, 25.7 kDa) contains a high proportion of β-sheets structures (34%) compared with the α-helix content (9%).[9] It was shown that trehalose is more efficient than other disaccharides (sucrose and maltose) to stabilize proteins in aqueous solutions against high temperature.[10,11] Consequently, the influence of trehalose on the stability of LYS and CHYM was analyzed *in situ* during each stage of a freeze-drying cycle.

It was shown[3] that micro-Raman spectroscopy was very suitable for monitoring protein denaturation *in situ* during a freeze-drying cycle, using a pressure–temperature chamber. Micro-Raman spectroscopy gives the opportunity to analyze, simultaneously, freezing, ice sublimation, and water desorption and the correlated structural changes in proteins, in the presence or absence of bioprotectant solutes. Important information on the protein–ice–sugar interactions and about the influence of trehalose on the protein stability during the different stages of a freeze-drying cycle can be expected from these analyses.

MATERIALS AND METHODS

Materials

Lysozyme, α-CHYM, and heavy water (isotopic purity of 99.990% atom D) were purchased from Sigma–Aldrich (St Louis, MO) and used without further purification. High-purity anhydrous trehalose was supplied from Sigma and freeze-dried with a 10-wt % sugar/D_2O solution to replace O-H (OH)by O-D (OD) groups by isotopic exchange. In the absence of trehalose, formulations for freeze-drying were prepared by dissolving proteins in D_2O (10 wt %) at $T = 23°C$. In the presence of trehalose (T), 10, 20, and 30 wt % freeze-dried (T10, T20, and T30)/D_2O mixtures were first prepared (at $T = 23°C$ for T10 and T20, at $T = 35°C$ for T30), and thereafter, the protein was added while the temperature was slowly decreased down to $T = 23°C$. A protein concentration of 10 wt % relative to the weight of the sugar–water mixture was systematically used. The solution was agitated in Eppendorf agitator at 23°C for 1 h to ensure complete isotopic exchanges between T and D_2O mixtures and the surface of the native protein.

Instruments

Raman investigations were carried out using a Renishaw InVia Raman spectrometer (Renishaw plc, Wottonunder-Edge, Gloucestershire, UK), composed of a single-grating spectrograph coupled with an optical Leica microscope (Leica microsystèmes, SAS, Nanterre, France). The 514.5-nm line of a Modu-Laser (Modu-Laser, Centerville, UT) argon laser was used for excitation. Focusing the laser beam via a ×50 long-working distance objective, a volume of about 100 μm^3 was systematically analyzed. The spectra were collected in backscattering geometry, with a resolution of about 2 cm^{-1} in the 600–3100 cm^{-1} spectral range.

In situ monitoring of the protein structure during a freeze-drying cycle was possible using a THMS 600 Linkam pressure–temperature device (Linkam Scientific Instruments, Guildford, Surrey, UK). About 450 μL of protein formulations were loaded in a cylindrical quartz cell.

Freeze-Drying Procedure

A freeze-drying cycle, schematically plotted in Figure 1, was roughly followed in most of the experiments performed with LYS, β-lactoglobulin (BLG) and CHYM proteins dissolved in 0, 10, 20, and 30 wt % trehalose–D_2O mixtures. Figure 1 shows temperature and pressure in the three typical process segments: (1) freezing, (2) primary drying, and (3) secondary

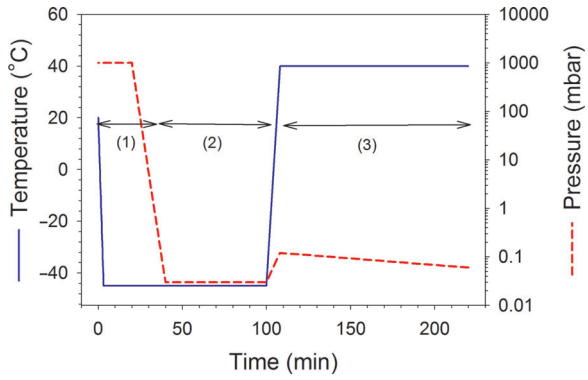

Figure 1. Temperature and pressure variations during the three stages of a lyophilization cycle. (1), (2), and (3), respectively, correspond to freezing, primary drying, and secondary drying.

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