Temperature Scanning FTIR Analysis of Secondary Structures of Proteins Embedded in Amorphous Sugar Matrix

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ABSTRACT: Heat-induced changes in secondary structures of five proteins (bovine serum albumin, BSA; human serum albumin, HSA; myoglobin; ribonuclease A, RNase A; and, β-lactoglobulin, β-Lg) in an amorphous sugar matrix were analyzed by temperature-scanning Fourier transform infrared spectroscopy to elucidate the mechanism of heat-induced conformational change of solid-phase proteins. Three sugars, trehalose, maltose, and dextran (MW 6000), were used. Loss of α-helices due to increasing temperature was observed for BSA, HSA, and myoglobin, which are rich in α-helices. RNase A showed a marked decrease in predominant secondary structural components (β-sheet) with increasing temperature. However, no noticeable changes in the content of secondary structures, except for a slight loss of α-helices, were observed for β-Lg, which is also β-sheet-rich. These heat-induced conformational changes were significant at temperatures above the glass transition temperature. The heat-induced conformational change in BSA dried with sugar appeared time-independent and was clearly different from that due to dehydration and from the thermal conformational change for a solution of BSA. In particular, differences in secondary structural components that increased due to loss of α-helices were noted. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:3088–3098, 2009

Keywords: FTIR; thermal denaturation; secondary protein structure; amorphous sugar; glass transition; hydrogen bond

INTRODUCTION

Conformational change of proteins is a primary degradation pathway of protein-based pharmaceuticals.[1,2] Temperature is one of the main factors determining the stability of proteins, and, thus, the degree of protein conformational change. Therefore, the impact of a wide range of temperature change to the conformational change of protein has been extensively investigated. Circular dichroism (CD) spectroscopy[3–5] and differential scanning calorimetry (DSC)[5–8] of protein solutions enable *in situ* analysis of conformational changes and denaturation induced by alterations in temperature. Secondary structures present at different temperatures can be measured using a temperature-controlled CD spectrometer.[3–5] DSC analysis measures changes in specific heat of proteins associated with thermal denaturation.[5–8]

However, protein-based pharmaceuticals often exist in solid, dried matrices formed by stabilizers and excipients and are subjected to high temperature in some cases, which includes spray drying. Proteins in aqueous solution are dried to

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prolong shelf life and improve handling properties. The above-mentioned CD is unavailable for conformational analysis of dehydrated proteins. DSC also provides no detailed information on what conformational changes of a protein occur due to heating, although DSC will detect protein thermal denaturation in a dried solid, as well as in a solution. Thermal conformational changes of solid-phase proteins have not been studied in detail. Fourier transform infrared (FTIR) spectroscopy is frequently used to analyze secondary protein structures.[5,9–11] IR absorption bands, corresponding to C=O (amide I band), N–H (amide II), and C–N (amide III) of peptide linkages, are resolved into multiple component bands corresponding to different secondary structures.[5,9–11] By controlling the sample temperature, changes in secondary structures of proteins embedded in dried solids due to alterations in temperature can be analyzed using IR-spectroscopy.

In this study, we conducted a temperature-scanning Fourier transform infrared (TS-FTIR) spectroscopic analysis[12] of proteins embedded in an amorphous sugar solid using a temperature-controlled FTIR spectrometer. Five types of proteins and three types of sugars were studied. Protein samples (containing sugar) were dehydrated under a stream of nitrogen gas and then under vacuum. The protein sample dried with sugar was heated gradually, and the IR spectra of the sample were measured at various temperatures. The secondary protein structures at different temperatures were determined by analyzing amide I bands in the IR spectra. Heat-associated conformational changes in amorphous sugar matrix-embedded proteins were compared with those of protein in aqueous solution. Differences in the mechanism of thermal conformational change among different types of sugar and protein are discussed.

MATERIALS AND METHODS

Materials

Trehalose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) and α-maltose (4-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dextran (Mr 6000 from *Leuconostoc sp*.) was obtained from Fluka Chemie GmbH (Buchs, CH, Switzerland). Five proteins, bovine serum albumin (BSA, A-7638), human serum albumin (HSA, A-8763), β-lactoglobulin (β-Lg, L-0130), myoglobin (MyoG, M-0630), and ribonuclease A (RNase A, R-5500) were obtained from Sigma–Aldrich Co. (St. Louis, MO). All proteins were used without further purification.

Preparation of Amorphous Sugar–Protein Mixtures

Aqueous solutions containing 10 mg/mL sugar and 1–10 mg/mL protein were prepared. A 10-μL droplet of the sugar–protein solution was placed in the center of a BaF_2 disc (32 mm diameter × 3 mm thickness) and dehydrated at room temperature in a stream of nitrogen (~10 L/min) for 20 min and subsequently under vacuum for 1 day. Furthermore, the obtained sugar–protein sample was stored for 3 days over P_2O_5 under vacuum at 25°C to remove all water. Complete dehydration of the protein-sugar mixture was confirmed by comparing the intensities of IR bands due to O–H stretching vibration (~3400 cm^{-1}) for samples that had been desiccated over P_2O_5 for different periods of time. It was also confirmed that the IR band, as a result of hydration water, was diminished to an undetectable level by dehydration under the same conditions using an amorphous sugar sample (protein free). In addition, a sugar-free protein solution (50 mg/mL BSA) was prepared and thoroughly dehydrated using the procedure described above.

TS-FTIR Analysis

TS-FTIR analyses were performed as previously described.[12,13] In brief, a BaF_2 disc, with a dehydrated sugar–protein mixture, was placed in a SpectraTech Heated Cell (Shelton, CT) and then heated at a rate of 1.0°C/min from room temperature (25 ± 5°C) to more than 150°C. The sample temperature was monitored and controlled using an ES100P digital controller (OMRON Co., Kyoto, Japan) that had been calibrated using a previously prepared calibration plot.[12] During heating, IR spectra of the sample were collected at appropriate intervals using a Nicolet Magna 560 FTIR (Nicolet, Madison, WI). For each IR spectrum, 64 scans were conducted over the range of 650–4000 cm^{-1} with a resolution of 2 cm^{-1}. When necessary, bands arising from water vapor were subtracted from the measured spectrum in an interactive manner using OMNIC software, version 4.1a (Nicolet). At least two