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Controlled water content for evaluation of denaturation temperature of freeze-dried enzymes

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ABSTRACT

Water content and storage temperature play an important role in determining the thermal stability and activity retention of biomolecules, mainly in freeze-dried proteins; for this reason, the study of the effect of both properties on the thermal stabilization of proteins is paramount to the biopharmaceutical industry. A parameter used to evaluate thermal stability of proteins is the denaturation temperature (T_d), reported extensively for a large number of proteins. Although it is known that T_d values are strongly dependent on moisture, water content is not necessarily considered or deliberately controlled during denaturation measurement for solid samples. In the present work a method to evaluate T_d under controlled water (ca. 6.5%) was implemented and tested for solid lysozyme to overcome the limitations encountered during the determination of T_d. The implemented method can be used for quality control purposes for solid protein products since it allows an objective comparison of samples differing in provenance.

1. Introduction

Proteins and therefore all protein based pharmaceutical products are sensitive to three major processes of degradation: formation of aggregates, denaturation by temperature and hydrolysis [1]. To inhibit degradation of proteins by hydrolysis, biopharmaceutical and biotechnological industries use freeze-drying as a process to obtain products as dried powders [2,3]. Since most of the commercial products are stored as dried solids it is important to directly monitor the structural and thermal stability of dried biomolecules to fully characterize whether a protein with poor storage stability is present or not in the solid state [4,5].

Among the parameters that can be monitored in solid state is denaturation temperature, T_d, which is related to thermal stability of proteins. This parameter is normally measured calorimetrically by heating a protein and monitoring the temperature to detect endothermic processes associated with thermally induced denaturation. A protein is a molecule composed of a large number of amino acids, linked together through strong peptide bonds (primary structure); the side chains of these amino acids can establish van der Waals interactions, hydrogen bonds and saline bridges providing the protein with a characteristic conformation (secondary structure). In addition, proteins also adopt a three-dimensional folded shape (tertiary structure) where the hydrophobic groups are folded towards the interior and the hydrophilic portion to the exterior. When a protein is subjected to heating, the chemical forces that maintain the integrity of the protein are disrupted and intermolecular interactions broken, leading to an endothermic conformational change that causes the protein to unfold; this denaturation process can involve the loss of secondary and tertiary structure [6,7]. Since this process of conformational distortion of the protein requires a transfer of heat it is possible to detect the denaturation temperature as the maximum in the peak of the endothermic process; this value is reported as denaturation temperature (T_d).

T_d is a parameter extensively used to analyze thermal stability for a large number of protein based biomolecules (enzymes, hormones, antibodies, etc.). As an example, Fig. 1 shows a summary of the values of T_d reported for lysozyme, an enzyme commonly used as a model of study. As can be seen, T_d is strongly dependent on water content, particularly when the amount of water is below 25% of the mass of the sample. During the denaturation process at low water contents, as the number of water molecules is reduced, folded and unfolded states of the protein are structurally modified due to the hydrogen bond interactions between water molecules

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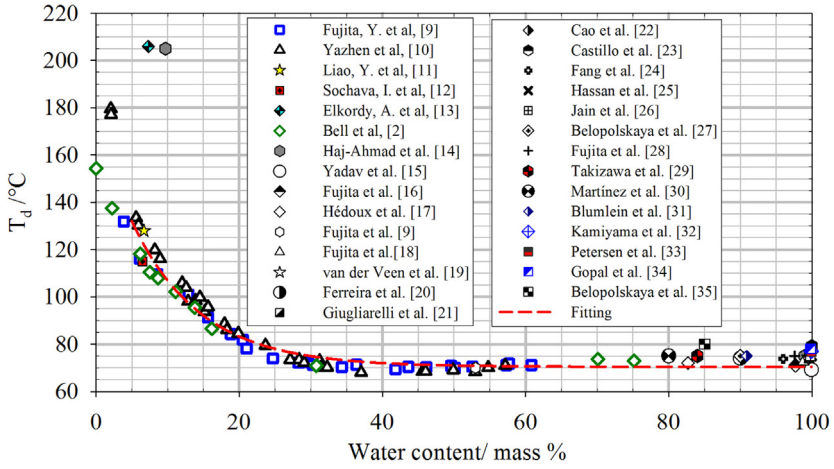

Fig. 1. Denaturation temperature of lysozyme, T_d, in solid state and in solution (above 25%). Fitting T_d values reported for water content, x, between 5 and 99.5 mass% with non-linear regression leads to the function $T_d/°C = 103.9e^{-0.1046x} + 70.4$ (see dashed line). Experimetal data were taken from references [2] and [9–35].

and protein shifting towards stiffer protein–protein interactions leading to a decrease in the denaturation enthalpy, a decrease in the denaturation entropy and therefore, an increase in the denaturation temperature. Above 25% water, the molecular water/enzyme ratio is approximately 260 and does not have a significant effect on denaturation temperature since these water molecules provide all the interactions that might be present in the solution [8,9].

Fig. 1 also shows that for solid samples with water contents below 10% there are significant discrepancies in the temperatures of thermal denaturation. Table 1 presents in better detail the T_d values reported for native lysozyme in solid state. These discrepancies may be due to the fact that most freeze-dried protein based products are in the amorphous state and measuring water contents below 10% can be difficult since some water molecules are so tightly bound that these can be considered an integral part of the protein. Another reason for the discrepancies is that the denaturation enthalpy is also dependent on water content in solid samples. Values of enthalpy of denaturation decrease as water content decreases; at very low levels of hydration, the endothermic denaturation process is difficult to detect calorimetrically. Fujita et al. [9] have reported that protein molecules in the samples with very low water content do not completely unfold because polar amino acid residues compete with water to form hydrogen bonds and this can cause a decrease of the enthalpy of protein denaturation.

Taking into consideration the difficulties of measuring the denaturation process of solid protein samples at low hydration values, the purpose of this work is to introduce controlled water content as a necessary condition for the measurement of the heat induced denaturation temperature of a protein. Even though there are many studies that have demonstrated the influence of water on the thermal denaturation of proteins (see the references included in Fig. 1) this information has not yet been used to facilitate or standardize measurement.

The present work presents the limitations encountered during determination of thermal denaturation of commercial solid samples. For samples with very low water contents (2–4%), T_d cannot be detected since the calorimetric denaturation signal is very small; on the other hand, freeze dried products can easily absorb water when exposed to ambient atmosphere for short periods of time provoking variations in the T_d values measured. These changes are an impediment to objective comparison of thermal stability of samples with different provenance. To overcome these difficulties, standardization of the measurement of T_d under fixed water control was implemented with freeze-dried lysozyme as a model.

2. Experimental

2.1. Materials

Lysozyme from chicken egg white (lyophilized power, L6876) was purchased from Sigma Aldrich and used without further purification. Humidity controlled baths were prepared by dissolving NaOH (Fermont) in distilled water. Concentrations of NaOH for the solutions with specific values of water activity were selected according to Fontana et al. [36].

2.2. Preparation of solid lysozyme samples with different water contents

To simulate samples of different provenances, solid samples of lysozyme with different water contents were prepared by three different methods: (a) free-standing solid samples (exposure to ambient atmosphere at different relative humidities) (b) water elimination by freeze-drying of enzyme aqueous solutions and (c) controlled water sorption of powder solid samples. Freeze dried lysozyme with low water content (less than 2%) directly as received from the provider was also analyzed. Details of preparation of samples for each process are described as follows.

2.2.1. Free standing samples (exposed to ambient atmosphere)

To demonstrate direct sorption of moisture from ambient, freeze dried lysozyme samples were placed in aluminum DSC cells and exposed to ambient atmosphere for 30 min on two different days with different relative humidity (RH). After the exposure time, cells were sealed and samples were analyzed.

2.2.2. Freeze drying method

To prepare lysozyme with different water contents (in this case by water elimination), a solution of the enzyme was prepared by mixing unprocessed lysozyme with water (0.2 g L^{-1}). After the solution was prepared, samples of 40 μL of the lysozyme solution were placed in aluminum DSC pans. The purpose of using these containers was to freeze dry samples directly on the containers where the denaturation process would be evaluated by thermal analysis. Once sample solutions were placed in the aluminum pans they were frozen at −80 °C and then transferred to a Labconco® freeze-dryer. The temperature and pressure of the freeze-dryer was maintained at −50 °C and 0.015 mbar. After preliminary measurements, freeze drying times of 0.6, 1, 3, 15 and 72 h were selected to obtain samples with different water contents.