An Equation to Line the Borders of Folding-Unfolding Transition Diagram of Lysozyme

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ABSTRACT

It is important for the formulators of biopharmaceuticals to predict the folding-unfolding transition of proteins. This enables them to process proteins at predetermined conditions without denaturation. Depending on apparent denaturation temperature (T_m) of lysozyme, we have derived an equation describing its folding-unfolding transition diagram. According to water content and temperature, this diagram was divided into three different areas which are the area of the water-folded lysozyme phase, the area of the water-folded lysozyme phase and the bulk water phase, and the area of the denatured lysozyme phase. The amount of water content controlled the appearance and intensity of Raman band at ~1787 cm^{-1} when lysozyme powders were thermally denatured at temperatures higher than T_m.

INTRODUCTION

Protein structure and dynamics are determined by the intraprotein and protein-water non-covalent interactions.[1,2] Hydration degree of proteins, which is the weight ratio of water to protein, should be ~0.2 to initiate protein function, moreover, full protein function requires a hydration degree of ~1.[3] However, proteins are in their solid state during pharmaceutical processes and storage. Water contents of protein powders affect their stability and performance.[4] Water content alters the mechanisms of proteins' degradation reactions, and this alteration also depends on other conditions such as temperature and the formulation compositions.[5] In general, increasing water content decreases the stability of protein powders. Water acts as a plasticizer which enhances the molecular mobility of the proteins and so leads to an increase in their degradation rates. This shortens the shelf-life of solid protein powders and formulations.

Many studies have used lysozyme as a model protein to understand the effect of the water content on protein stability using differential scanning calorimetry (DSC). Previous works used sealed DSC pans to study the effect of water content on the glass transition temperature (T_g) of lysozyme, they found that T_g of lysozyme decreased by increasing the water content to approach a plateau level.[6,7] Similarly, increasing water content within lysozyme powders decreased their apparent denaturation temperature (T_m) in sealed DSC pans.[8] Therefore, both water content and temperature line the borders between the native (folded) and denatured (unfolded) states of lysozyme powders. These borders are important to predetermine the processing conditions (water content and temperature) which preserve the native structure of proteins during manufacturing processes (e.g., spray drying). Here, we derive an equation correlating water content of lysozyme powders with their $T_m s$, and then use this equation to line the borders of the folding-unfolding

transition diagram of lysozyme. Also, we use Raman spectroscopy to monitor water content effect on the molecular conformation of lysozyme upon the thermal unfolding transition of lysozyme powders.

EXPERIMENTAL METHODS

Materials. Lyophilized hen egg-white lysozyme powder (Biozyme Laboratories, UK) was purchased and considered to be unprocessed lysozyme powder.

Sample Preparation. The unprocessed lysozyme powder was used to prepare a series of lysozyme powders with different water contents either by raising or reducing their water content. The water residues were manipulated by exposing the powders to humidified air or to anhydrous nitrogen gas for different times at 30 °C. The prepared powders were immediately sealed and left to equilibrate at room temperature at least for a weak after preparation. This time is enough to ensure uniformity of water distribution in lysozyme particles.[9] The prepared series of lysozyme powders were tested to correlate the water content with T_m. To monitor the water content effect on the molecular conformation of lysozyme upon the thermal unfolding transition, the lysozyme powders with different water contents were thermally denatured inside hermetically sealed pans by heating to temperatures higher than their unfolding transition peaks. These samples were considered to be the thermally denatured lysozyme powders.

Thermogravimetric analysis (TGA). The water content of each powder was determined using Thermo Gravimetric Analysis (TGA) (Perkin Elmer Ltd., UK). Samples (5-10 mg) were heated from 30 °C to 210 °C at a scan rate of 10 °C/min in an aluminum pan under nitrogen flow at 20 ml/min. Each sample was analyzed in triplicate. The decrease in the weight before

decomposition was considered as water content. TGA results were validated by re-analyzing the water content of some samples using Karl Fischer Titration (KFT) (701 KF Titrino with 703 Ti stand, Metrohm, Switzerland). Using TGA instead of KFT as only a few mg is enough for TGA.

Differential scanning calorimetry (DSC). T_m of each sample was measured using Differential Scanning Calorimetry (DSC) (Perkin-Elmer Ltd., UK). To study the effect of water residue on the thermal stability of lysozyme (the value of T_m), the evaporation of water during heating was suppressed by using Large Volume Capsules, which are stainless steel sample containers equipped with an O-ring seal to provide a hermetic seal. Lysozyme powders (5-10mg) and solutions in water (20 µl) were encapsulated in the hermetically sealed pans and scanned between 40 °C to 200 °C at 5 °C/min in triplicate. DSC was also used to prepare the thermally denatured lysozyme powders by heating them inside the hermetically sealed pans to temperatures higher than their unfolding transitions.

FT-Raman spectroscopy. FT-Raman spectra of the lysozyme powders at different water content and their thermally denatured lysozyme counterpart powders were recorded with a Bruker IFS66 optics system using a Bruker FRA 106 Raman module. The excitation source was an Nd: YAG laser operating at 1064 nm and a laser power of 50 mW was used. The FT-Raman module is equipped with a liquid nitrogen cooled germanium diode detector with an extended spectrum band width covering the wave number range 1850-1100 cm^{-1}. Samples were placed in stainless steel sample cups and scanned 200 times with the resolution set at 8 cm^{-1}. The observed band wave numbers were calibrated against the internal laser frequency and are correct to better than ±1 cm^{-1}. The spectra were corrected for instrument response. The experiments were run at a controlled room temperature of 20 ±1°C.

Results and discussion

We employed this protein because it folds in a highly cooperative manner and so exhibits an all-or-none thermal unfolding transition, and so it displayed one denaturation peak, in solid and solution states,[10,11] which makes the study easier. The driest prepared lysozyme powder contained 2.6±0.1 %w/w water and its T_m was 436.9±0.6 K, while the wettest powder contained 20.3±2.3 %w/w water and its T_m was 355.5±3.1 K. In Figure 1 is a DSC thermogram of each sample, showing an inverse correlation between water content and T_ms.

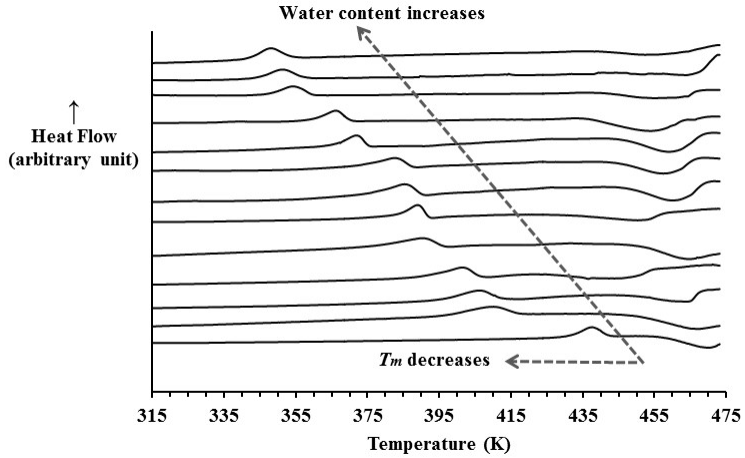

Figure 1. Differential scanning calorimetry thermograms of lysozyme powders (in sealed pans) at different water content.

Previous study demonstrated that DSC thermograms of lysozyme powders with different water content performed in pierced pans showed two endothermic peaks, the first broad peak, which extends from ~298 to 433 K, indicates water evaporation and its area directly correlates to the water content, the second peak at ~473 K refers to the protein denaturation (T_m) and its position

146 is independent of water content because water evaporates through the pierced pans before the
147 unfolding transition (denaturation) of lysozyme.[10]

148 However, unlike the experiments performed in the pierced pan, Figure 1 shows that the
149 position of the endothermic unfolding peak (T_m) varied for different water contents when DSC
150 experiments were performed under sealed conditions. The DSC thermograms (Figure 1) did not
151 show the water evaporation peak because the evaporation of water during heating was suppressed
152 by the hermetical seal, i.e., the pressure generated from increasing the temperature of the air, which
153 had been trapped during the capsulation of the hermetically sealed pans, prevented water loss.
154 Figure 1 also show an exothermic peak after lysozyme unfolding in all samples analysed with the
155 sealed pans. This peak was reproducible and showed little variation between samples, with a peak
156 position ranging from 453 to 468 K. It was believed that this exothermic peak could be due to the
157 aggregation of lysozyme molecules after unfolding, however re-scanning the sealed pans
158 containing previously aggregated lysozyme powders also gave an exothermic peak at the same
159 position but without the endothermic unfolding peak.

160 We hypothesized that water molecules associate with lysozyme powders to form a
161 homogenous water-folded lysozyme phase. This phase unfolds at a kinetic thermal energy
162 depending on water mole fraction (X_w) and lysozyme mole fraction (X_p) within the powders. When
163 X_w increases, T_m of the unfolding kinetic energy is reduced, and its reduction stops when the water
164 molecules reach their saturation limit within the water-folded lysozyme phase. Above this limit,
165 water molecules segregate from the water-folded lysozyme phase to form another phase of bulk
166 water, and so they cannot reduce T_m further.

167 X_w / X_p equals N, the ratio of water molecules to lysozyme molecules, and it is calculated
168 according to Eq. (1):

$$N = 14296 \times \mathrm{W\%}/18 \times \mathrm{P\%} \quad (1)$$

18 and 14296 are the molecular weights of water and lysozyme, respectively, and W% and P% are the percentage of water weight and protein weight in the powders, respectively. The results were tabulated in Table 1.

Table 1. The apparent denaturation temperature (T_m), number of water molecules associating with one lysozyme molecule (N), water mole fraction (X_w) and lysozyme mole fraction (X_p) of lysozyme powders at different water content.

Water content %w/w	T_m (K)	N	X_w	X_p
2.5 (0.1)	436.9 (0.6)	20.4	0.9532	0.0468
4.6 (0.3)	412.0 (1.6)	38.1	0.9744	0.0256
5.2 (0.1)	406.2 (2.1)	43.4	0.9775	0.0225
6.4 (0.2)	401.9 (1.4)	54.3	0.9819	0.0181
7.7 (0.1)	390.3 (0.2)	66.6	0.9852	0.0148
8.5 (0.1)	388.8 (2.6)	73.8	0.9866	0.0134
9.2 (0.5)	385.5 (0.3)	80.5	0.9877	0.0123
9.9 (0.1)	383.5 (0.6)	87.7	0.9887	0.0113
12.0 (0.1)	371.8 (0.4)	108.7	0.9909	0.0091
14.4 (0.1)	365.7 (0.4)	133.1	0.9925	0.0075
15.6 (0.6)	364.0 (1.6)	146.6	0.9932	0.0068
20.3 (2.3)	355.5 (3.1)	201.8	0.9951	0.0049

Values within parenthesis are standard deviation, n = 3.

T_m reflects the value of thermal kinetic energy needed to overcome the cohesion energy stabilizing the folded state of proteins. This cohesion energy results from Van der Waals attraction, electrostatic interaction, hydrogen bonding, and conformational entropy (hydrophobic effect).[12] Electrostatic and hydrophobic interactions play the main roles in protein folding.[1,13] Water affects these interactions and hence the magnitude of T_m needed to unfold proteins. Water molecules surround the surface of protein molecules and penetrate into their cavities.[14] Surrounding water molecules strengthen the hydrophobic effect by an increase in the entropy of water due to association between hydrophobic residues.[13] However, according to Coulomb's law, the interior

183 waters weaken the electrostatic forces because they increase the dielectric constant in the interior
184 of protein molecules and also increase the distances between polarized and ionized groups of
185 proteins.[15,16] Our results demonstrated that the net effect of water weakens the forces responsible
186 for the folded state, and so the increased water content facilitates the protein unfolding at lower
187 T_m. Dehydration of lysozyme powders stabilizes its native form because the free energy change of
188 the lysozyme denaturation increases by dehydration.[17]

189 Multiplying the gas constant (R) by T_m gives the kinetic thermal energy needed to unfold
190 lysozyme. Interestingly, our data (Table 1) prove that RT_m and $\ln N$ correlate linearly with a
191 correlation coefficient close to one ($r = -0.997$), and their linear equation is:

192 $$\ln N = \text{Slope}\ RT_m + \text{Intercept} \qquad (2)$$

193 Figure 2 shows the straight line of Eq. (2) which was regressed in Eq. (3).

194 $$\ln N = -\frac{RT_m}{72.3} + 15.0 \qquad (3)$$

195 where 72.3 has units of Cal.mole^{-1} and 15.0 has units of mole.mole^{-1} (i.e., unitless). R = 1.9872041
196 Cal.mole^{-1}.K^{-1}, therefore Eq. (3) can be reduced to Eq. (4):

197 $$T_m = -36.4 \ln N + 545.7 \qquad (4)$$

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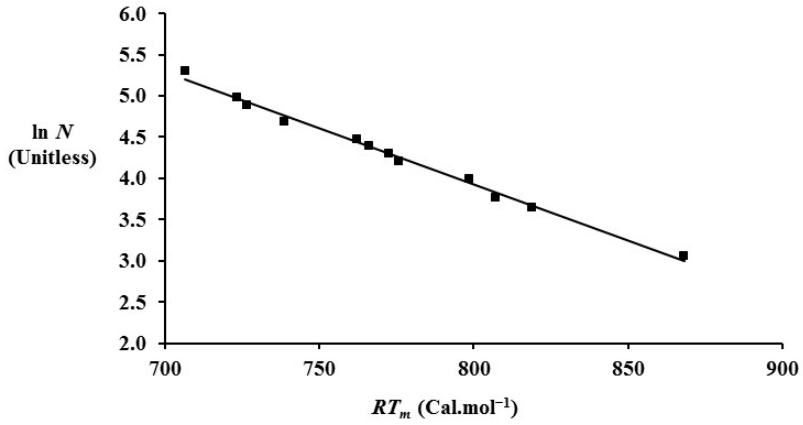

Figure 2. The linear correlation between the denaturation thermal kinetic energy and logarithm of water molecules associating with one lysozyme molecule.

We measured T_ms of lysozyme solutions containing 80 %w/w and 70 %w/w of water. T_ms of the solutions were 348.2±0.7 and 348.0±1.0 K, respectively, and they were similar (T-test: $P < 0.05$). According to our hypothesis, T_m of the lysozyme solutions equals the apparent denaturation temperature of the saturated water-lysozyme phase (T_m^S). Therefore, we extrapolated Eq. (4) to T_m^S (348K), which is the same as that of the solutions, to calculate the number of water molecules which saturate one molecule of lysozyme (N^S) as follows:

$$T_m^S = -36.4 \ln N^S + 545.7 \qquad (5)$$

The calculated N^S was 228 water molecules according to Eq. (5). This water amount is equivalent to 22.3 %w/w and 0.3 g/g protein. We then prepared another lysozyme powder with a water content of 23.2±1.1 %w/w, which is similar to the calculated N^S. T_m of the prepared water saturated powder was 347.8±1.4 K, which is similar to T_m^S (T-test: $P < 0.05$). Therefore, we used temperature and N to draw the folding-unfolding transition diagram of lysozyme. Figure 3 shows this diagram in which the denaturation line obeys Eq. (3) until reaching the water saturated solid state at N^S. After this value of water molecules, T_m did not significantly change. Our calculated value of N^S, i.e. 228 water molecules, is similar to previously reported values of the hydration shell of lysozyme. Previous researchers used gigahertz to terahertz spectroscopy[18,19] Raman spectroscopy[20] neutron and X-ray scattering[21] NMR[22] to quantitate the hydration shell of lysozyme, and these methods estimated similar values of the hydration shell of lysozyme, and the values ranged between 170-270 water molecules. Hydration shell molecules have different properties from bulk water.[23] Our results demonstrate that the effect of water molecules on T_m changed at $N > N^S$. Therefore, we speculate that water molecules begin to form the bulk water at $N > N^S$. Therefore, the folding-unfolding transition diagram of lysozyme (As shown in Figure 3) was divided to three areas; (A) represents the area of the water-folded lysozyme phase, (B)

represents the area of the water-folded lysozyme phase and the bulk water phase, and (C) represents the area of the denatured lysozyme phase, and the line separating (C) form (A) and (B) is the denaturation line.

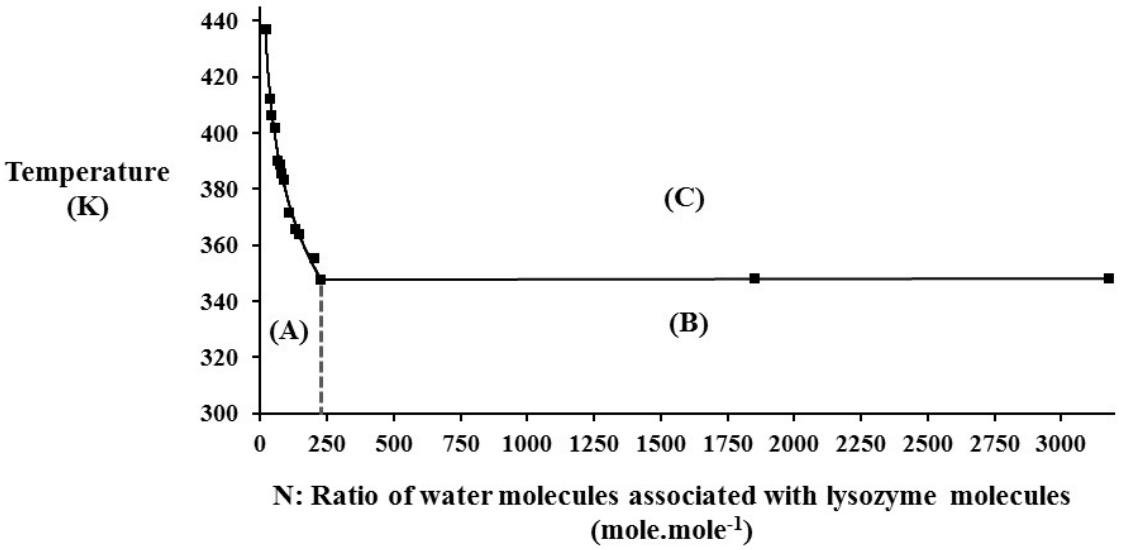

Figure 3. The folding-unfolding transition diagram of lysozyme. (A) represents the area of the water-folded lysozyme phase, (B) represents the area of the water-folded lysozyme phase and the bulk water phase, and (C) represents the area of the denatured lysozyme phase; the line separating (C) form (A) and (B) is the denaturation line.

Although T_m of lysozyme solutions is usually ~348 K, it changes due to additives (e.g., co-solvents, pH, ionic strength).[10,24,25] Also, additives changed T_m of lysozyme powders.[8] We could conclude that if the additives are miscible with water, they will participate in forming water-protein phases (in both solid and solution states). Therefore, they would change the constants of Eqs. (3) and (5). Whilst other proteins may have different constants.

Previous Raman study clarified the significant effects of hydration degree of native lysozyme powders on the back bone and the side chain conformations of lysozyme.[9] In other words, this previous study covered the molecular conformation of lysozyme under the denaturation

235 line of the folding-unfolding transition diagram of lysozyme. Here, the molecular conformation of
236 lysozyme above the denaturation line were investigated. Raman spectra of the thermally denatured
237 powders at different hydration degree and their original powders were collected at room
238 temperature and presented in Figure 4. The peak intensities of the spectra were normalized using
239 the band at 1447 cm^{-1} (CH bending) as internal intensity standard. This is because its intensity and
240 position are unaffected by changes induced in protein structure after applying different stresses.[26]
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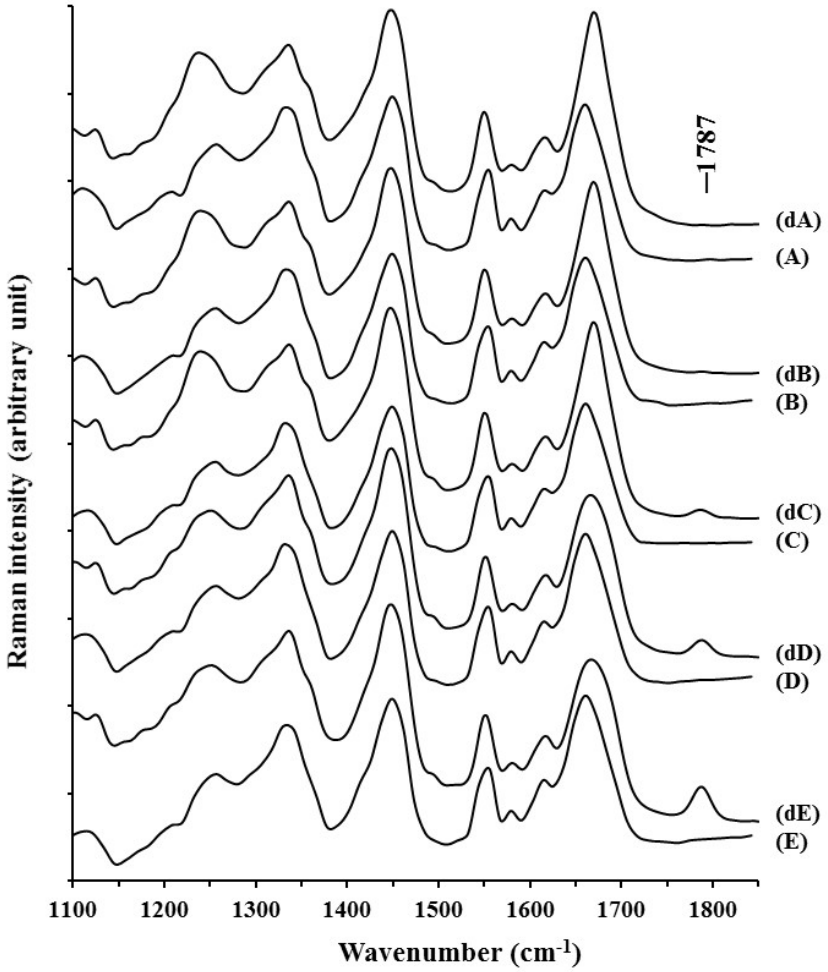

Figure 4. Representative Raman spectra of lysozyme powders in the 1100−1850 cm^{-1} wavenumber region, (A), (B), (C), (D) and (E) represent lysozyme powders at different water content of 20.3, 12.0, 8.2, 5.2 and 2.5 %w/w, respectively, and (dA), (dB), (dC), (dD) and (dE) represent their thermally denatured counterpart powders (in sealed pans), respectively.

Within the investigated wavenumber region (1100−1850 cm^{-1}), we did not notice significant differences between the studied original lysozyme samples (Figure 4, spectra A, B, C, D and E). However, compared to the original lysozyme powders, the Raman spectra of the counterpart thermally denatured powders showed changes in the the peak positions of amide I and amide III bands. The peak of amide I was upshifted from ~1660 to ~1671 cm^{-1} after thermal denaturation (Figure 4, spectra dA, dB, dC, dD and dE), and also spectra dD and dE show broadening in the amide I band in the case of the thermally denatured samples at low hydration degree (5.2 and 2.5 %w/w). The peak of amide III was downshifted from ~1256 to ~1249 cm^{-1} at low hydration degree (spectra dD and dE), and it was further downshift to ~1240 cm^{-1} with an increase in the intensity at higher hydration degree (spectra dA, dB and dC). The Raman Bands of the amide I (C=O stretch) and amide III (N-H in-plane bend + C-N stretch) are indicators of the protein's secondary structures.[9] The mid-point of amide I is expected to be at 1665±5 cm^{-1} for α-helix and 1670±3 cm^{-1} for β-sheet and disordered secondary structure, and the mid-point of amide III is expected to be at 1267±8 cm^{-1} for α-helix, 1235±5 cm^{-1} for β-sheet and 1245±4 cm^{-1} for disordered structure.[27,28] Therefore, the changes in Raman bands (presented in Figure 4) indicate that the content of the β-sheet and disordered secondary structure were increased and at the same time the α-helix content was decreased after the thermal denaturation. Similar changes in the positions of amide I and amide III Raman bands of lysozyme powders were reported when lysozyme powders were denatured using mechanical stress[10] and γ-radiation[29], and the researchers attributed these changes to a decrease in the α-helix with an increase in the β-sheet and disordered structures. However, in the case of the thermally denatured powders, a new Raman band at ~1787 cm^{-1} appeared, and its intensity decreased by raising the water content (Figure 4, spectra dC, dD and dE), and the peak disappeared at water content ≥ 12 %w/w (Figure 4, spectra dA and dB).

Therefore, the appearance of this peak at 1787 cm^{-1} relates to the nature of lysozyme molecules' movement during the thermal unfolding transition and this movement depends heavily on the amount of hydration degree of the powders. The new band at 1787 cm^{-1} could be assigned to the stretching of free hydrogen bonding carbonyl group. As the line of amide I at wavenumbers ≥ 1685 cm^{-1} is expected to be assigned to the disordered secondary structure without hydrogen bonding.[27] It can be concluded that the higher the water content in lysozyme powders during unfolding transition, the higher the freedom for lysozyme molecules to move and saturate the free carbonyl groups of the peptide bonds resulted from the unfolding transition. At water content ≥ 12 %w/w, the lysozyme molecules have enough freedom to move and saturated all the free carbonyl groups, and this explains the disappearance of the Raman band at 1787 cm^{-1}. This finding agrees with previous results stating that at water content between 11 and 21 %w/w, lysozyme possess a glasslike dynamic transition from rigid to flexible state due to the mobility increase of the structural units.[30]

Therefore, the shape of amide I Raman band, the position and intensity of amide III Raman band, and the intensity of the Raman band at ~1787 cm^{-1} of the thermally denatured powders depend on the water content of their original native powders. This indicates that the molecular structure of the thermally denature lysozyme depends on its hydration degree.

This folding-unfolding transition diagram of lysozyme was obtained using sealed pans in order to ensure mass conservation of the water-lysozyme system. Therefore, it was determined at constant volume but variable pressure. Fortunately, in our case, we capsulated the sealed pans at atmospheric pressure (~0.1MPa), and according to the general gas law, the increase in the pressure due to the temperature increase from 298 to 473 K inside the hermetically sealed pans would reach

~0.2 MPa. This pressure values have a negligible role in the lysozyme denaturation. It was confirmed that lysozyme is denatured when pressure is at much higher values >500 MPa.[31]

CONCLUSIONS

These results allowed us to derive an equation correlating T_m of lysozyme with its hydration degree. T_m exponentially decreased when the hydration degree increased, and it did not significantly decrease further after hydration degree reaching a value similar to hydration shell of the lysozyme. We used the derived equation to draw the folding-unfolding transition diagram of lysozyme. This diagram will be useful to process lysozyme at predetermined conditions of temperature and water content without denaturation. The molecular conformation of the thermally denatured lysozyme was found to be dependent on the hydration degree of the original native powders. A new Raman band at 1787 cm^{-1} appeared in the spectra of the thermally denatured lysozyme, and its intensity reversibly correlated with the water content and it disappeared at the water content ≥ 12 %w/w.

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Notes

The authors declare no competing financial interest.

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$$T_m = -36.4 \ln N + 545.7 \text{ when } N \leq N^S$$

$$T_m \cong T_m^S \text{ when } N \geq N^S$$

T_m: Apparent denaturation temperature of lysozyme.

N: Ratio of water molecules to lysozyme molecules.

S: Superscript refers to saturated water-lysozyme state.

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