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Testing the Functionality of Lysozyme After Desiccation by Light Assisted Drying

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Abstract

Protein based therapeutics and diagnostics play an important role in modern medicine such as cancer treatment. The current method for storage of proteins is freeze drying, which is expensive and difficult. One major challenge faced by current preservation techniques is maintaining protein functionality during processing and storage. Light assisted drying (LAD) provides a novel method of preservation with potential for room temperature storage. LAD uses a near-infrared laser to illuminate and desiccate a protein suspended in a trehalose solution. The rapid desiccation of the solution by LAD forms an amorphous trehalose matrix for the preservation of the protein. The goal of this research was to test for any damage to the secondary and tertiary structures, or unfolding, of a test protein, lysozyme, after LAD processing. When a protein unfolds, it loses its functionality. Differential Scanning Microcalorimetry (DSC) was used to measure the unfolding temperature of lysozyme after LAD processing. The unfolding temperature of unprocessed lysozyme was compared with the unfolding temperature of LAD processed samples. The samples processed were 40µL drops of 0.50mg/ml lysozyme in 0.2M trehalose and 0.33X Phosphate Buffer Saline. The LAD processed samples were illuminated with a 1064nm laser in a chamber maintained at 11% relative humidity for 1 hour and the samples reached an average maximum temperature of 44.4 ± 0.7°C. The unfolding temperature of unprocessed lysozyme was measured at 73.26°C, and the LAD processed lysozyme unfolded at 73.24°C. The LAD process showed no significant effect on the unfolding temperature of lysozyme compared with an unprocessed sample. LAD is a promising method for the preservation of proteins for use in protein-based therapeutics and diagnostics.

Keywords: Lysozyme, Trehalose, Preservation

1. Introduction

Protein-based therapeutics and diagnostics play an important role in modern medicine. Protein therapeutics have been developed to treat diseases ranging from arthritis to cancer.[1] Proteins are also used in diagnostics, which contain immobilized capture proteins for the detection of diseases.[2] A challenge in the development of protein-based diagnostics and drugs is maintaining the protein in the folded state during processing and storage as the three-dimensional structure of the protein is often responsible for its functional activity.[3] The current method for storage of proteins is freeze drying (known as lyophilization), which is expensive and difficult. Even after lyophilization, many proteins still need to be stored below 4°C. Protein therapeutics and diagnostics would benefit from a quick, relatively inexpensive processing method that could also enable higher temperature storage. We are developing a light-based drying technique to preserve biologics in a trehalose sugar matrix that can be stored at supra-zero temperatures.

Recent research has demonstrated that anhydrous, or dry state, preservation in a trehalose amorphous solid matrix may be an alternative to freeze drying for the preservation of biological samples.[4,5] We have previously described a new processing technique, light assisted drying (LAD), to create trehalose amorphous solids for the preservation of

biologics.[6] LAD uses illumination by near-infrared laser light to assist in the formation of trehalose amorphous solids. Static air-drying of sugar solutions is dominated by evaporative cooling which causes the drying rate to slow substantially and allows for crystallization of the sugars. Crystallization puts physical stress on embedded biologics that can lead to damage. LAD selectively heats water to overcome cooling due to evaporation and speeds dehydration of the samples. As water is removed from the sample, the remaining sugars and salts become concentrated, and, as long as the solutes do not crystallize, the viscosity increases with progressive water loss until an amorphous solid is achieved. Because a substantial reduction of molecular mobility is necessary to ensure an extended shelf life, samples generally need to be stored below the glass transition temperature, T_g, of the trehalose matrix to prevent degradation.[7] Below T_g the trehalose maintains its amorphous state. Trehalose is ideal as it can form an amorphous solid at high temperatures and acts as a bioprotectant. The Gordon-Taylor equation can be used to predict the glass transition temperature (T_g) of trehalose-water mixtures,

$$T_g = \frac{x_1 T_{g,1} + k_{GT}(1-x_1)T_{g,2}}{x_1 + k_{GT}(1-x_1)} \tag{1}$$

where the glass transition temperatures of pure trehalose and pure water are given by $T_{g,1}$ and $T_{g,2}$ respectively, x_1 is the weight fraction of trehalose, and k_{GT} is an empirically determined fitting parameter of 5.2.[8] The glass transition temperature for an amorphous trehalose solid depends on the water content of the sample after processing. The more water that remains in the sample, the lower the glass transition temperature. Lower moisture contents are necessary for storage at higher temperatures. Our previous work has demonstrated that LAD can reach end moisture contents (EMCs) low enough for storage at elevated temperatures in the glassy state and determined the optimal LAD processing parameters for achieving these EMCs.[6]

The goal of this research was to determine if LAD processing affects the functionality of an embedded protein. Differential Scanning Calorimetry (DSC) was used to determine if LAD denatured the protein lysozyme.

2. Methods

A schematic of the experimental setup is shown in Fig. 1. An IPG Photonics continuous wave (CW) ytterbium fiber laser at 1064 nm (YLR-5-1064) was used for LAD processing. The source had a maximum power output of 5 W with built in control of power. The laser emits a collimated, single-mode, Gaussian beam with a full width at half maximum (FWHM) spot size of ~4.5 mm which was measured using a BeamTrack 10A-PPS thermal sensor (Ophir Photonics). A FLIR SC655 infrared (IR) camera was used to record the temperature of samples during all processing. Thermal imaging provides a method to noninvasively monitor the sample temperature during processing. The camera (sensitive from 7.5 to 14 microns) has an array of 640x480 pixels and a maximum frame rate of 200 fps. All studies were performed in a humidity-controlled environment that was kept at approximately 11% relative humidity (RH). This was achieved by pumping dry air into a chamber containing the experimental setup as shown in Fig. 1 and monitoring the RH with a temperature and RH logger (ONSET UX100-011).

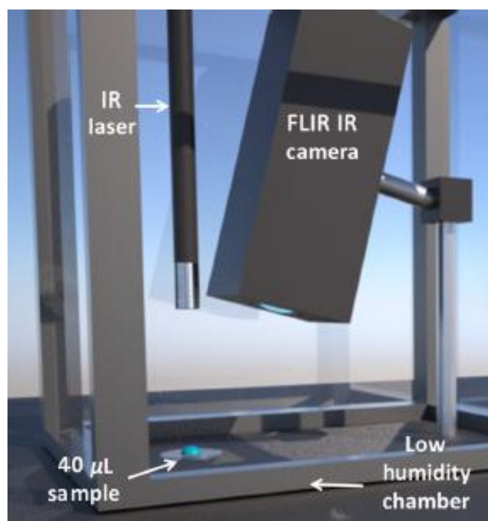

Fig. 1 LAD setup with 1064nm laser, FLIR infrared camera, and 40μL sample inside a low relative humidity chamber.

All samples in the studies consisted of 40 µl droplets containing a model protein, egg white lysozyme (Worthington Biochemical LS002933), dissolved in drying solution (DS) at a concentration of 0.50 mg/ml. This was verified using the absorption of light at 280 nm with a microplate spectrophotometer (Bio-Tek Synergy HT). The DS consisted of 0.2M disaccharide trehalose in 0.33 x phosphate buffer solution (PBS).[9] The dry weight of the DS was determined through bake out method to be 7.01% the mass of a sample. Dry weight was adjusted to include the mass of the protein based on its concentration to determine the total dry weight.

For each test, a 40 µL droplet of the protein/drying solution was deposited onto a substrate and the initial mass was determined gravimetrically using a balance (RADWAG AS 82/220.R2) accurate to 0.01 mg. The substrates used in these studies were 18mm diameter borosilicate glass coverslips (Fisherbrand 12-546). The glass cover slips allow for easy recovery and rehydration of the proteins after LAD processing. On the glass coverslips, the samples were droplets roughly 2 mm in thickness with a diameter of approximately 7 mm. The sample was then moved into the humidity chamber for laser irradiation. Samples were processed for 60 minutes at 5 W (26.9 W/cm^2). The maximum temperature of the sample was monitored during processing using the thermal camera. Samples reached a maximum sample temperature of 44.4 ± 0.7°C during LAD processing. After irradiation, the sample was removed from the humidity chamber and immediately massed again. End moisture content (EMC), which is a measure of the amount of water relative to the dry mass of a sample, was calculated as:

$$EMC = \frac{m_f - m_s - m_{dw}}{m_{dw}} \tag{2}$$

where m_f is the mass of the final sample including the mass of the substrate, m_s, and m_{dw} is the calculated dry weight of the initial sample. All samples in this study were processed to an average EMC of 0.14 ± 0.04 gH_2O/gDryWeight.

For comparison, 40 µL samples of the same solution were air-dried by placing the samples in the humidity-controlled chamber (~11% RH) for 60 minutes. The EMC's for these samples were calculated using equation (2) in the same manner as used for the LAD processed samples. Air-dried samples had an average EMC of 5.11 ± 1.30 gH_2O/gDryWeight after processing. Air drying for 60 minutes did not result in the formation of an amorphous or crystalline trehalose solid.

A MicroCal VP-DSC microcalorimeter (MicroCal, Northampton,MA) was used to assess the effect of LAD processing on the thermal stability of the protein lysozyme. For comparison, four samples of lysozyme were tested for unfolding using DSC. Unprocessed lysozyme was used as a control and LAD processed lysozyme was used to determine if laser irradiation caused unfolding of the protein. Air dried samples and samples subjected to a 43°C water bath (the approximate maximum temperature of samples during LAD processing) were also include for comparison.

The main compartment of the DSC is shown in Fig. 2, where S is the sample cell and R is the reference cell. The sample cell was filled with the sample to be tested, and the reference cell was filled with the same solution without

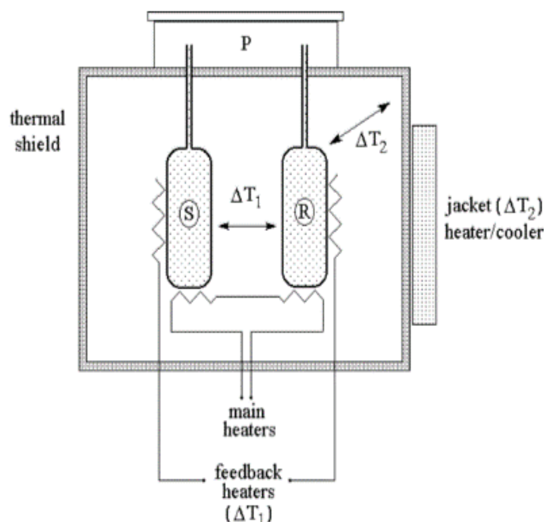

Fig. 2 Differential Scanning Calorimeter with sample cell S and reference cell R.[10]

lysozyme. As the DSC heats up the sample and reference cells at a specific rate, the energy required to maintain the same temperature between them is measured. Since the unfolding of a protein is an endothermic process, information about the unfolding can be measured through the heat energy supplied to the sample cell relative to the reference cell.

The LAD processed (N=2) and air-dried (N=2) samples were rehydrated with deionized water after desiccation for DSC measurement. The resulting concentration due to dilution for all samples was 0.20 mg/ml of lysozyme in 0.085M trehalose and 0.14X PBS. The concentration of the solutions after dilution was measured using a BioTek Synergy HT Microplate reader (BioTek instruments, Winooski , VT). The 43°C water bath (N=3) samples were 0.20 mg/ml lysozyme in 0.085M trehalose and 0.14X PBS. These samples were placed in a test tube then submerged in a water bath at 43°C for 1 hour. This temperature was similar to the peak temperatures that samples reached during LAD processing.

DSC baseline repeatability was established with a minimum of 5 reference solution scans (0.085M trehalose in 0.14X PBS). Once a stable baseline was established, the samples containing lysozyme were loaded by syringe into the calorimeter. All scans were carried out at a scan rate of 90°C/hr from 10°C to 90°C with a 15 minute pre-scan equilibration time. The data was analyzed using Origin software provided by MicroCal to determine the midpoint temperature of transition, T_m, and the calorimetric enthalpy of unfolding, ΔH.

3. Results

The calorimetric curves of the four samples described in Sect. 2 were obtained using DSC. The data was normalized based on the concentration of lysozyme. Fig. 3 shows representative calorimetric curves of the samples and the

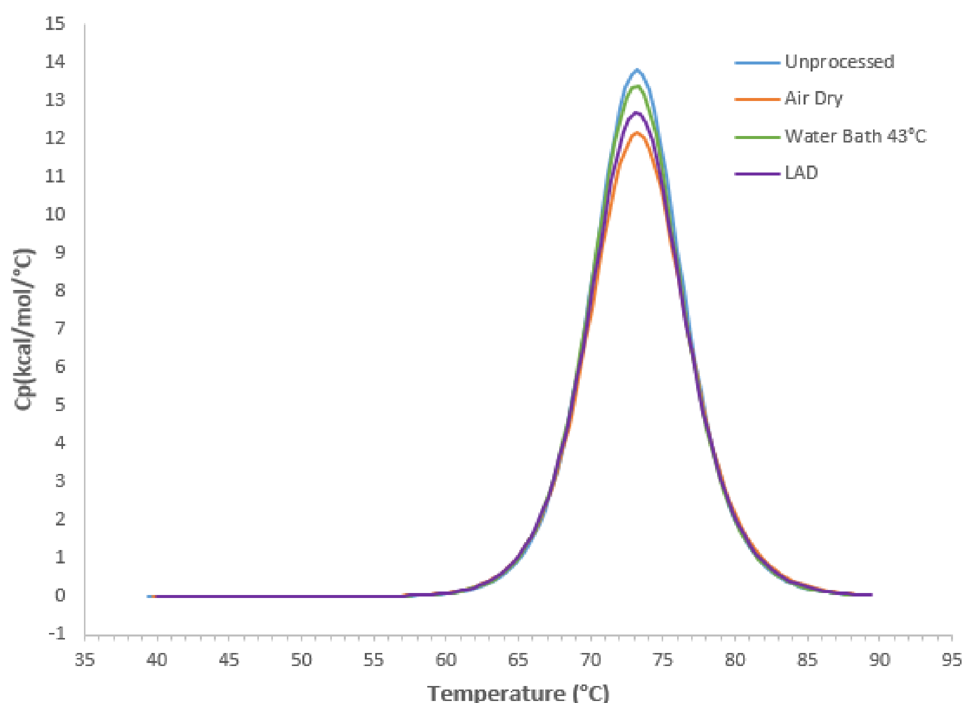

Fig. 3 Calorimetric curves of LAD processed, unprocessed, air dried and thermally processed lysozyme.

derived thermodynamic properties are given in Table 1. The percent difference for T_m and ΔH as compared to the unprocessed lysozyme are included in Table 1. The changes in T_m and ΔH when compared to the unprocessed control sample are negligible for LAD processing, as well as thermal processing at 43°C and air drying. LAD has little/no effect on the functionality of lysozyme.

Table 1. Thermodynamic parameters for lysozyme as determined with DSC

	T_m (°C)	Change in T_m[a]	ΔH (kcal/mol)	Change in ΔH[a]
Unprocessed (N=3)	73.20 ± 0.06	---	116 ± 2	---
Air Dried (N=2)	73.29 ± 0.01	- 0.12%	106 ± 3	8.6%
43°C Water Bath (N=3)	73.18 ± 0.02	0.03%	112 ± 1	3.4%
LAD (N=2)	73.19 ± 0.02	0.01%	108 ± 2	6.9%

[a]Change in thermodynamic parameters compared to the control (unprocessed) sample.

4. Conclusion

The goal of the experiment was to determine if the LAD process denatured the protein lysozyme. Previous testing of protein functionality after LAD processing was done using an assay described by Worthington Biomedical.[6] The assay found that LAD did not affect the functionality of lysozyme. The data in Table 1 shows only small deviations in T_m and ΔH, between LAD processed and unprocessed lysozyme. This data shows that LAD processing did not

significantly unfold lysozyme, which agrees with the findings of the assay. Future work with LAD will be to optimize the drying process and test the functionality of lysozyme and other proteins after processing in LAD with different setups. LAD is a more attractive option for anhydrous preservation when compared to lyophilization and other preservation techniques because it is cost effective, relatively fast, and enables monitoring of sample temperature as well as precise control over energy deposition.

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