MOISTURE SORPTION BEHAVIOR, STABILITY, AND PROTEIN FORMULATION CHARACTERISTICS OF A TREHALOSE CHOLINE CHLORIDE EUTECTIC COMPOSITION

by

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ABSTRACT

TIFFANY ERIN BURGIN. Moisture sorption behavior, stability, and protein formulation characteristics of a trehalose choline chloride eutectic composition. (Under the direction of DR. GLORIA D. ELLIOTT)

Protein based drugs and therapeutics are an important treatment for many life-threatening diseases but they can also be extremely expensive. The unique 3-dimensional structure of a protein is responsible for its function but also contributes to its sensitivity to environmental factors such as moisture, temperature, and pH. The challenges of manufacturing, storing, and delivering modern protein therapies require better solvents that increase manufacturability, extend shelf-life, and reduce cost. Recently, low melting point sugar-salt mixtures called Natural Deep Eutectics (NADES) have been explored as alternative solvents and stabilizers for proteins. These compositions are relatively easy and inexpensive to prepare and have the benefit of being in the liquid state at room temperature. The current study focuses on compositions based on trehalose, a disaccharide commonly used for stabilizing proteins, and choline chloride, a salt frequently used to formulate NADES. Solubility and thermal stability of the protein lysozyme were investigated as a function of water content for compositions with a trehalose:choline chloride molar ratio of 1 to 4. This ratio of components forms a clear liquid at room temperature with a very low moisture content and is the ratio used for similar sugar-salt eutectic compositions. The effect of varying the moisture content on the thermal stability of the model protein lysozyme was measured by differential scanning calorimetry. As the amount of water in the composition decreased, lysozyme

showed an increase in thermal unfolding temperature, indicative of increased thermal stability.

Experience has shown that changes in the water content can cause NADES compositions to become unstable and form undesirable crystals. Using saturated solutions with controlled humidity values, a sorption isotherm of the composition was constructed, and the compositions were monitored for evidence of crystallization at each tested relative humidity. The samples were observed for crystal formation over a period of approximately 3 weeks and weighed every few days to determine moisture uptake and to identify when samples achieved moisture equilibrium. The trehalose choline chloride composition remained stable and free of crystals when the moisture content was maintained between 0.1 and 0.2 grams of water per gram dry weight, corresponding to relative humidity values between 23% and 33%. Pure amorphous trehalose is characterized by a relative low level of absorption at low humidity values consistent with monolayer adsorption, with a transition to multilayer absorption at higher humidity values. The addition of choline chloride to the composition changed the isotherm to one that is characterized by a steady increase in moisture content over the entire humidity range without an observable monolayer sorption region. The shift in the sorption isotherm suggests that in the NADES composition the moisture is diffusing and distributing evenly throughout the bulk of the material. Lysozyme stored in this composition at controlled humidity levels of 23% and 33% for 5 weeks and diluted back into water, exhibited melting temperatures and enthalpies of unfolding consistent with freshly prepared protein solutions.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ASTM	American Society for Testing and Materials
BET	Brunauer Emmett Teller
DSC	Differential Scanning Calorimetry
FTIR	Fourier Transform Infrared Spectroscopy
IL	Ionic Liquid
NADES	Natural Deep Eutectic Solvents
RH	Relative Humidity
TCH	Trehalose Choline Chloride

CHAPTER 1: INTRODUCTION

1.1. Protein Based Drugs and the Need for New Solvents

As the availability of protein based drugs and therapeutics grows, so does the need for new biocompatible solvents[1-2]. Protein based drugs differ from traditional small molecule drugs in that there is a need to maintain the protein in its folded state throughout the manufacturing, storage, and delivery processes. The unique three-dimensional structure of a protein is responsible for its function but also contributes to its sensitivity to environmental factors such as moisture, temperature, and pH. When a protein is removed from its native environment, it often becomes unstable and is susceptible to denaturation. Lyophilization (freeze-drying) has been used successfully to prolong the shelf life of many proteins, including therapeutics, but proteins in this state have bioavailability challenges when administered orally and must be reconstituted prior to delivery by injection in liquid form. Due to protein-solvent interactions, many liquid formulations are stable only for a few days, which increases the complexity of administering these drugs and leads to waste of expensive therapies. Liquid formulations that can be stored for 1-2 years, would reduce waste and make these therapies much more economical and accessible.

When designing liquid formulations, excipients are typically chosen for their ability to increase solubility of the protein or to prevent damaging events such as denaturation, aggregation, or oxidation. The list of approved excipients for

pharmaceutical use is limited to a few sugars, amino acids, polymers, salts, and detergents[3]. Some groups have explored low melting point (<100°C) organic salts, known as ionic liquids (ILs), as potential alternatives solvents for enzyme-based processes[4] and have demonstrated the ability of ILs to stabilize proteins[5-9]. ILs are polar liquids, made of large organic salts, with almost negligible vapor pressure, and they demonstrate the ability to solvate a variety of compounds[3]. Specifically, ILs have been shown to increase protein solubility or to prevent the denaturation and aggregation of proteins[2]. Part of the appeal of ILs is the ability to manipulate properties by varying the side chain length of the cation or altering the type of anion. High cost of the components and concerns about cytotoxicity of ILs have pushed researchers toward more natural substances such as organic acids, amino acids, sugars, choline, and urea[10]. When combined in specific ratios, these substances form low-melting point compositions with similar properties and design flexibility as ILs.

1.2. Deep Eutectic Compositions and Their Role as Protein Solvents and Stabilizers

Eutectic compositions, while similar to ILs in that they are polar solutions, are formed by combining a specific ratio of components such that it solidifies or melts homogenously. Deep eutectic compositions are so named because they have a melting point significantly lower than that of the individual components. These non-volatile, low melting point compounds share many favorable properties with ILs[10-11], but have the advantage of being cheaper, less-toxic, and relatively easy to prepare[12]. Desirable properties, such as formulations that are biocompatible and remain liquid at room temperature, could greatly reduce the costs associated with sensitive protein based drugs. Many of the components used to form deep eutectic compositions are found naturally in

organisms that are freeze and drought tolerant. Common components include glucose, sucrose, fructose, trehalose, lactic acid, malic acid, glycerol, choline chloride and urea[10]. Many of these sugars or acids are produced when the organism experiences stress and it is thought that these compositions prevent the formation of damaging ice crystals at low temperatures and function as preservatives in dry environments[13]. These compositions are often referred to as NADES (natural deep eutectic solvents) due to their presence in living organisms. NADES are especially interesting since their properties can be tailored, much like ILs, by selecting salts with different cations and anions, as well as varying the component ratios, or by adding small amounts of water to obtain a liquid at room temperature[1, 12].

This study focuses on compositions based on the disaccharide trehalose and the salt choline chloride. It is well established that trehalose has a stabilizing effect on proteins[14-16] though it has not been studied well as part of the spectrum of NADES compositions. Trehalose works as a stabilizer for proteins by maintaining a glassy state in the surrounding solution or by binding with polar sites on the protein, though the mechanism varies depending on the solvent and protein considered[17-18]. This disaccharide has also been studied by our lab in the past for its glass forming ability and for those reasons it was chosen as the hydrogen bond donor for this study[19-20].

The choline chloride salt chosen for this study has been used frequently in the literature to form eutectic compositions with low melting points[11, 13, 21-22]. The reduction in melting point results from a weakening of the ionic network. Interactions between the cation and the hydrogen bond donor reduces the anion-cation electrostatic force and, in

turn, lowers the freezing point[22]. The interactions between the salt and hydrogen bond donor also affects the solubility of proteins.

A previous study with trehalose choline chloride (TCH) compositions reported that compositions of TCH with molar ratios of 1:4 and 1:3 yielded clear liquids and showed signs of hydrogen bonding in their FTIR spectra[21]. In that study, the FTIR spectra of the TCH compositions was compared to the spectra of trehalose and choline chloride alone. They observed changes in frequency shifts, peak widths, and absorbance values that indicated an increase in hydrogen bonding. Hydrogen bonding plays a role in some of the unique characteristics that NADES compositions exhibit, low melting points, for instance. The increase in hydrogen bonding weakens the ionic network and increases the stability of the liquid state. This study also reported that dilutions of the TCH mixtures with up to 25% water by weight maintained the additional hydrogen bonds, while those with 50% water or more behaved more like aqueous solutions. This study is focused on a 1:4 molar ratio of trehalose to choline chloride as that ratio forms a clear liquid at room temperature and is the ratio used most frequently cited in the literature.

Protein solubility in any solution depends largely on the pH of the solvent and the distribution of hydrophilic and hydrophobic amino acids on the surface of the folded protein[23]. A protein's structure begins with the sequence of amino acids, which twist or fold into alpha helices and beta sheets. The polypeptide chains then fold and the three-dimensional structure emerges. Most proteins fold in such a way that the hydrophobic regions are turned inward and the hydrophilic regions face outward making them soluble in water. The goal in engineering solvents is to create an environment in which it is thermodynamically favorable for the protein to remain folded in solution. If proteins are

not soluble in a composition, there is the possibility of aggregation and the solutions may not preserve the protein structure. As new solvents are explored, the pH, water content, and polarity of the solvent will be important in predicting how it will interact with a specific protein.

1.3. Moisture Sorption and Its Relationship to Solution Stability

In addition to its effects on protein solubility and stability, the water content of the solvent also influences the long-term stability of the formulation. NADES have already shown promise as potential solvents for stabilizing proteins so it is important to consider how the solutions might behave during extended utilization or storage[10, 21, 24-26]. Moisture content can greatly affect the long-term stability of solutions, but so far there has been virtually no mention of water content in the NADES literature nor how water content was controlled. Hygroscopic salts, such as choline chloride, readily absorb moisture from the air and Choi, 2011 notes that certain eutectic compositions containing choline chloride exist with 6% water that does not easily evaporate but relatively little attention has been devoted to understanding and controlling the moisture content of NADES [13]. The tendency to absorb and retain water could affect the stability of the compositions.

Moisture sorption characteristics depend on the properties of the specific composition and can be characterized based on the shape of the equilibrium moisture curve, known as a sorption isotherm. This relationship between the moisture level in the sample and the moisture content in the environment is determined by placing samples in various relative humidity (RH) environments and allowing them to reach moisture equilibrium, where the partial vapor pressure in the sample is equal to the RH of the environment. The partial vapor pressure is directly correlated to water activity, a_w, which

is always a quantity between zero and one. Sorption isotherms can be determined for a variety of liquids and gases, but for this study only water vapor sorption was considered. Isotherms are dependent on temperature and whether the samples are adsorbing, taking on water, or desorbing, losing water, as well as the physical state of the sample, amorphous or crystallized. The difference in curves obtained for adsorption and desorption is known as hysteresis and is illustrated in Figure 1. The extent of hysteresis varies for different compositions.

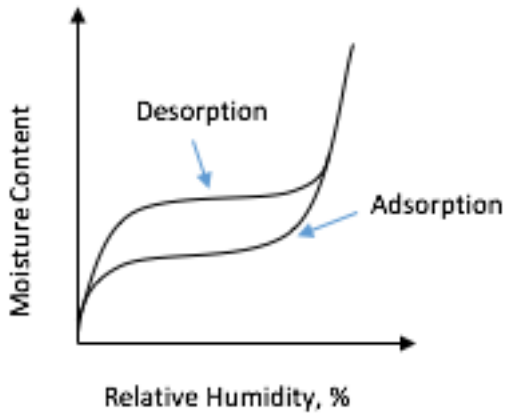

Figure 1. Hysteresis of sorption isotherms

Brunauer and his colleagues described five types of isotherms, shown in Figure 2 [27]. Type 1, known as a Langmuir isotherm, is associated with monomolecular adsorption by a porous solid. The Langmuir model is based on the assumption of identical, independent sorption sites[28-29]. As shown in the figure, the initial moisture uptake happens quickly, then slows and levels off as the surface has adsorbed its limit. Type 1 isotherms indicate adsorption that is limited primarily to the surface of the sample.

The initial uptake of a Type 2 isotherm is similar in shape to a Type 1 isotherm and corresponds to monolayer adsorption. The flat middle section of a Type 2 isotherm

represents the saturation of the sample surface/monolayer. The difference between Type 1 and Type 2 isotherms, however, is that once the monolayer forms on a Type 2 substance, the adsorbate begins to penetrate the sample and multilayer absorption begins. Type 2 isotherms are typical for soluble products, where the vapor being absorbed coats the surface and once a certain vapor pressure is reached, the adsorbing component diffuses throughout the sample.

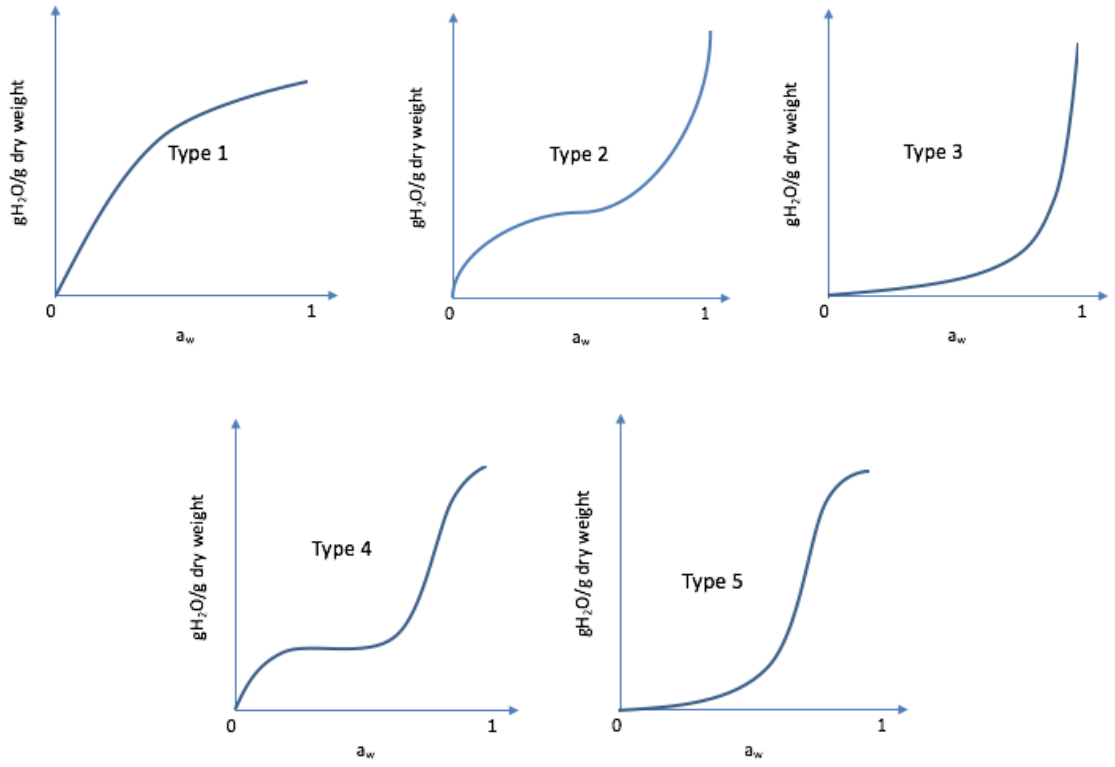

Figure 2. Sorption isotherm types, a_w = water activity

Type 3 isotherms indicate multilayer absorption only and are common for solvents. The sample begins absorbing vapor throughout its volume without the initial monolayer formation typical of a Type 1 or Type 2 isotherm. Type 3 isotherms are known as Flory-Huggins isotherms. The Flory-Huggins approach to understanding vapor

absorption into amorphous solids was to describe adsorption in terms of thermodynamics, treating the absorption process like a dissolution process[30]. In the 1940s, Flory and Huggins independently developed equations for polymer solutions that assume a process driven by minimum free energy requirements[31-32]. They found the free energy of mixing, ΔG_m, could be expressed by:

$$\frac{\Delta G_m}{RT} = \frac{\phi_1}{r_1}\ln(\phi_1) + \frac{\phi_2}{r_2}\ln(\phi_2) + \phi_1\phi_2\chi_{12}\ . \qquad (1.1)$$

In this equation, ΔG_m is the free energy of mixing, R is the universal gas constant, T is the temperature, ϕ is the volume fraction of the component, r is the degree of polymerization, and χ_{12} is the Flory-Huggins polymer-vapor interaction parameter[30, 33]. In the Flory-Huggins model the χ parameter is a measure of the polymer-solvent compatibility. It reflects the nonideality of the system, with large values of χ indicating low interaction and low values indicating strong interaction[30].

The well-known Brunauer Emmett Teller (BET) equation is often used to model both Type 2 and Type 3 isotherms. While the Languir model assumes only monolayer adsorption and the Flory-Huggins model can only represent multilayer absorption, the BET model was built to include both. BET remains one of the most common models for fitting sorption curves and is given as:

$$\text{BET: } W(a_0) = \frac{W_B C_B a_0}{(1-a_0)(1+(C_B-1)a_0)} \qquad (1.2)$$

where $W(a_0)$ is the moisture content of the sample at water activity a_0, W_B is the monolayer value (a representation of the number of hydrophilic binding sites for water) and $C_B$is an energy constant[30, 34]. The free energy constant, C_B, is related to the difference of free enthalpy or chemical potential of the sorbate molecules in their liquid state and in the monolayer[34] and is represented by:

$$C_B = k \exp\left[\frac{(\Delta H_a - \Delta G_L)}{RT}\right] \quad (1.3)$$

where ΔH_a is the enthalpy of adsorption, ΔG_L is the enthalpy associated with condensation of multilayers as a liquid, and k is a measure of the entropy change associated with adsorption. The energy constant, C_B, is what differentiates between a Type 2 and a Type 3 isotherm with Type 2 isotherms having larger values of C_B compared to those of Type 3 [30].

Type 4 isotherms are similar to Type 2 isotherms in that there is an initial adsorption process that forms a monolayer, then the vapor begins to permeate and multilayer absorption occurs. Following the multilayer absorption, Type 4 isotherms reach a limit which may be due to capillary condensation. Water sorption for Type 2 and Type 4 isotherms typically happens in three stages. At low water activity, the moisture content in the sample is strongly bound. In the second region, water is loosely bound but not as free as pure water. The last region represents a solution with properties very similar to free water[28]. Type 5 isotherms reach a limiting value toward the end of the isotherm, similar to Type 4, but the monolayer adsorption is missing meaning it initially sorbs more like a Type 3 [27-28].

Water absorbed by amorphous solids acts as a plasticizer, lowering the glass transition temperature and increasing molecular mobility[30]. This increase in mobility could alter many physical and chemical properties, including the tendency to form crystals.

1.4. Aims of the Study

The aim of this work is to characterize the moisture sorption behavior, stability, and protein formulation of a trehalose choline chloride deep eutectic composition. Since water content is associated with crystallization behavior in sugars, a better understanding of the sorption behavior of these compositions could help identify compositions that are metastable or conversely are more likely to crystallize[35]. The lack of information in the literature about water content and moisture sorption properties of NADES presents an opportunity to advance the study of these compositions in a way that has not previously been done. This goal of this study was to determine the moisture sorption characteristics of deep eutectic compositions, and also to determine solution stability by observing which solutions form crystals and which remain clear. Before beginning to use these compositions as stabilizers for biologics, it is important to understand more about the persistence of the deep eutectic characteristics in a range or humidity conditions. Solubility of the model protein lysozyme in the trehalose choline chloride composition as a function of water content was determined and the effect of trehalose choline chloride on the thermal stability of lysozyme was measured. Following the sorption studies, lysozyme was stored for 5 weeks in TCH compositions with moisture contents that were determined to be stable and compared to the freshly prepared solutions to evaluate the

suitability of these compositions as a protein solvent across a range of working humidity conditions.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

Trehalose dihydrate high purity was purchased from Pfanstiehl Laboratories, Inc (Waukegan, IL); choline chloride and anhydrous potassium carbonate were purchased from Sigma Aldrich (St. Louis, MO); potassium acetate and sodium bromide were purchased from Alfa Aesar (Haverhill, MA); anhydrous lithium bromide, lithium chloride extra pure, and magnesium chloride were purchased from Fisher Scientific (Hampton, NH); sodium chloride was purchased from Amresco Inc (Solon, OH). Hen egg white lysozyme was purchased from Worthington Biochemical (Lakewood, NJ).

2.2. Preparation of Relative Humidity Jars

Saturated salt solutions were prepared in glass mason jars to achieve RH values of 7% (LiBr), 12% (LiCl), 23% (KCH_3CO_2), 33% ($MgCl_2$), 43% (K_2CO_3), 59% (NaBr), and 76% (NaCl) according to Greenspan (1976) and the ASTM Protocol E104-02[36]. Fifty milliliters of water were added to each jar, then the appropriate salt was added and stirred until a thick, slushy mixture with little free water formed. Additional water was added if the mixture became too dry. The jars were covered and stored in a cabinet for at least 24 hours before use to allow them to come to equilibrium. Holes the size of an HH314A Omega humidity temperature probe bored into the jar lids were used to check the relative humidity and were otherwise sealed with rubber stoppers as shown below in Figure 3.

Experiments were conducted at room temperature, approximately 21°C unless otherwise noted.

Figure 3. Relative humidity jar and measurement device

2.3. Sample Preparation for Sorption Studies

The TCH mixture was prepared gravimetrically in a glass vial. The choline chloride was weighed directly into the vial while the trehalose was weighed on paper and added to the vial as shown below in Figure 4.

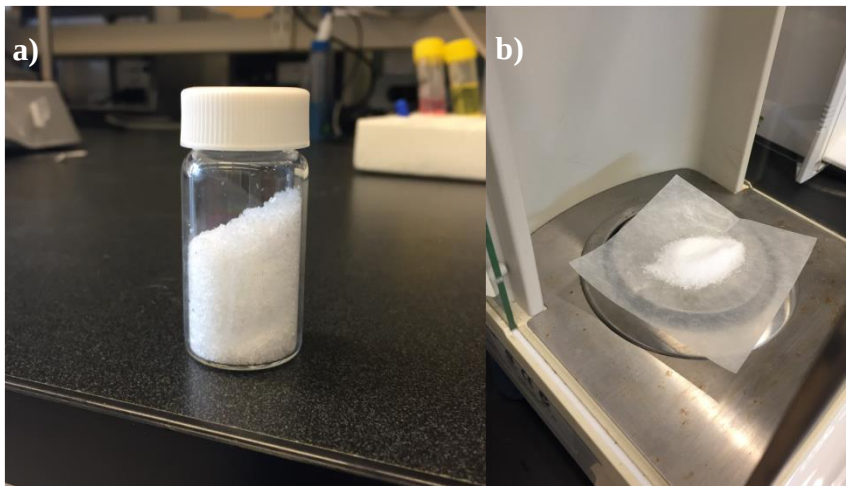

Figure 4. Dry samples of a) choline chloride and b) trehalose dihydrate

A 1:4 molar ratio of trehalose dihydrate and choline chloride was added to the vial with ten droplets of water. The sample was heated in a 75-80°C water bath with a stir bar until it became a clear, homogenous liquid[11]. If the sample did not become clear within a few hours, 5-10 additional drops of water were added. The sample solution was stored in the closed vial at room temperature until use.

2.4. Solubility Studies

Initial solubility studies were done with the model protein lysozyme in water to validate the method. Samples with known concentration were prepared by weighing the lysozyme and adding the appropriate amount of water to get the desired concentration in mg/mL. Samples were gently inverted several times and stored at room temperature for 1 hour to overnight to allow the protein to rehydrate. Solubility limits were determined via turbidimetric assay. For this determination, aliquots of 150µL were taken from each sample and added to a 96 half-well plate. Samples were then placed in the Biotek Synergy plate reader and absorbance was measured from 200 to 900nm. Lysozyme has a

characteristic fluorescence at 280nm that can be used along with Beer-Lambert's law to measure the concentration, however, for samples above 5mg/mL, the instrument sensor becomes saturated and the readings are not useful for determining concentration. To determine solubility limits, the absorbance at 600nm was used to indicate the presence of aggregate or particulate in suspension. At this wavelength, far from the characteristic absorbance, any increase in absorbance is an indicator that the protein is likely not completely in solution. A baseline was established by measuring the absorbance of pure water or TCH solution without any lysozyme. Samples were measured in triplicate and the averages plotted.

2.5. Sample Preparation for Protein Stabilization Studies

TCH solutions were prepared as described above. Water was added and the water content was measured using Karl Fischer titration until the desired solution was achieved. Lysozyme was weighed into the container and the appropriate amount of TCH was added to achieve a solution with a concentration of approximately 1mg/mL. Samples were gently inverted several times and left at room temperature for one hour or overnight to allow the lysozyme to rehydrate and go into solution. Some samples were dried by evaporative drying and rehydrated prior to scanning. Another sample of lysozyme in 0.1M NaAcetate buffer pH 4 was prepared for comparison.

To determine the stability of lysozyme in TCH over time, a set of samples were prepared at stable moisture contents, as determined from the sorption study, and stored at the appropriate humidity at room temperature for a period of five weeks. These samples were prepared with a protein concentration of 10mg/mL prior to storage. After five

weeks, they were diluted to approximately 1mg/mL and scanned using differential scanning calorimetry (DSC).

A Micro-cal VP DSC was used for determining the thermal stability of the protein. A baseline was established by scanning the TCH solution without protein at least three times until repeatability was evident. The TCH solutions used for the baseline scans are identical to the sample solutions only without protein. The baseline scans of the solutions without protein are done to remove any noise that might show up as a result of slight variation in the sample cells. Samples with protein concentrations of approximately 1mg/mL were added to the sample cell and scanned several times. Cells were rinsed with water and TCH solution between samples. The concentration of lysozyme in each sample was checked prior to scanning by reading the absorbance at 280nm on the Biotek Synergy plate reader. The exact concentrations were used to normalize enthalpy data. All samples were run in triplicate and the averages reported.

2.6. Moisture Sorption Experiments

For moisture sorption studies, small samples of the viscous TCH sample was pipetted onto petri dishes of known weight and dried by evaporative drying for 24-30 hours as shown in Figure 5. A glass coverslip was tared on the scale and 5-10% of the dried sample was transferred onto the coverslip. The coverslip with sample was added to a volumetric Karl Fisher titrator to determine the mass of water present and enable reporting of the percentage of water by weight. The remaining sample was weighed and placed in a relative humidity jar. Assuming a homogenous sample, the dry weight (anhydrous) was determined by subtracting the measured water content from the initial analyzed mass. Samples ranged from 500mg to 822mg dry weight, with most falling

between 500 and 700mg. Every few days, samples were briefly removed from the jars and weighed to determine the amount of water gained or lost. Samples were measured until crystals formed or until the mass equilibrated.

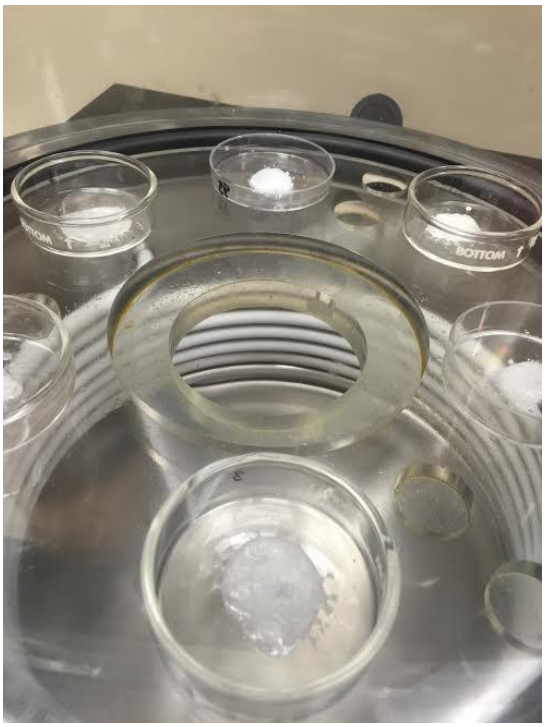

Figure 5. TCH samples in the evaporative dryer

Dry weight determinations were verified after samples equilibrated by bake-out in a gravity convection oven (VWR, West Chester, PA) at 95°C for 48 hours. Samples were then moved to a desiccator with phosphorus pentoxide and weighted inside a low humidity chamber until there was no change in weight, approximately one week. The dry weight measured by bake-out was used for calculating moisture content of the samples.

2.7. Data Analysis

The moisture sorption isotherm was plotted and fit to the standard BET model in OriginPro 8. Water content is expressed as either gH_2O/g dry weight or as weight percent water (gH_2O/mass sample). Sorption data is presented as the average of at least three samples; most are the average of more than five.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Solubility Studies of Lysozyme in TCH

The solubility of lysozyme in TCH formulations was investigated as a function of water content to determine the effect of TCH on the solubility of the protein lysozyme. Figure 6 shows the absorbance spectra of lysozyme at concentrations from 1mg/ml to 125mg/ml. There is a characteristic peak near 280nm that can be used can be used along with Beer-Lambert's law to determine concentrations, but the spectra is near zero away from this peak. For this reason, the absorbance at 600nm was used to indicate solubility challenges since and increase in absorbance at 600nm would suggest aggregation or settling of the protein. As shown by the blue line in Figure 7, in pure water, lysozyme showed no indication of solubility issues up to the maximum concentration tested, 200mg/ml. This is comparable to literature values, though there is some variation in the reported solubility of lysozyme in water[37]. When looking at this figure, it is important to note the scale. Compared to Figure 6 with peaks near 3, the highest absorbance measured in Figure 7 is 0.05. While the increase in absorbance is noticeable at this scale, it is for practical purposes zero.

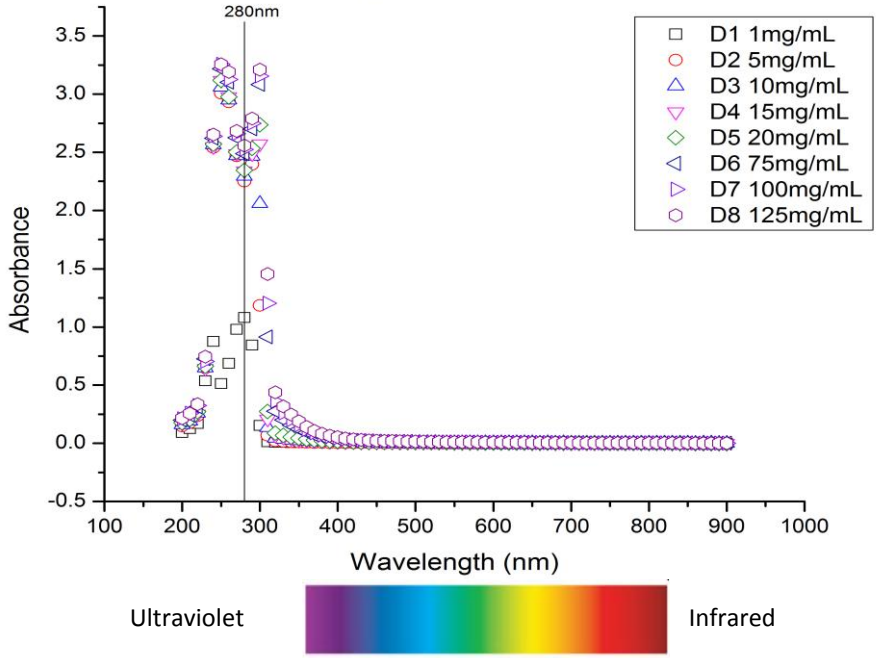

Figure 6. The absorbance spectra of lysozyme in water.

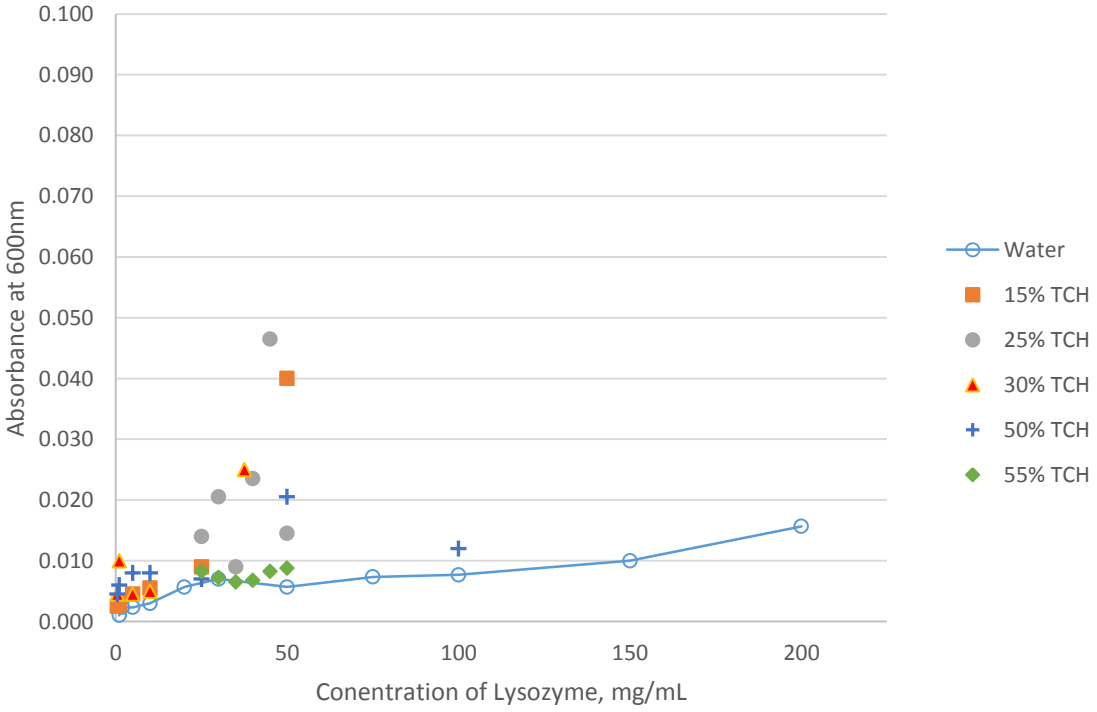

Figure 7. The absorbance values at 600nm for lysozyme in solutions with 0-55% TCH

The absorbance values are slightly higher in intermediate solutions with 15% and 25% TCH with small absorption increases observed at ~40mg/ml. For all the samples measured, the absorbance at 600nm was generally low, falling below 0.05, which suggests that the protein is soluble in these compositions. The concentrations that showed some increase in absorbance were well above the concentrations used in this study, so solubility issues were not a concern for any of the subsequent experiments. At concentrations below 40mg/mL absorbance values were low and comparable to those of lysozyme in pure water. The solution with the highest concentration of TCH, 55%, had some of the lowest absorbance values indicating that solubility of lysozyme may be better in these solutions. The results of the solubility study suggest that lysozyme is readily soluble in TCH solutions. If concentrations above 40mg/mL are desired, it would be necessary to conduct addition solubility studies.

3.2. Protein Stabilization Study

The stabilization effects of TCH on the model protein lysozyme were investigated to determine how the concentration of TCH affects the thermal unfolding temperature of the protein. The results of the stabilization study shown in Figure 8 are presented as a plot of the thermal unfolding temperature of lysozyme versus the weight percent of TCH in solution. Samples were run in triplicate. The thermal unfolding temperature of lysozyme increases with increasing concentrations of TCH indicating increased thermal stability of lysozyme in the solutions. The stored samples have a slightly higher unfolding temperature than a linear fit might predict, but still appear to follow the general trend. This suggests that there the thermal stability of the protein has not changed.

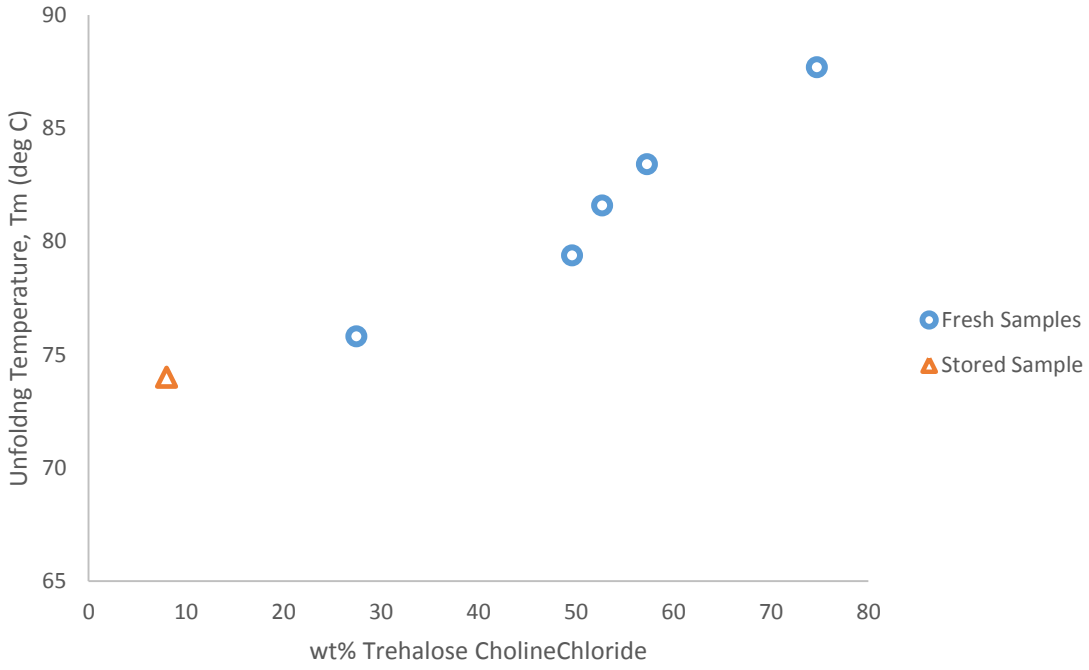

Figure 8. Thermal unfolding temperature, T_m, as a function of the weight percent of TCH

DSC thermal traces were fit in OriginPro with a non-two-state model that includes an output of both calorimetric enthalpy and Van't Hoff enthalpy, H_v. The calorimetric enthalpy is determined by the area under the curve and depends on the selected baseline as well as the normalized concentration. The Van't Hoff enthalpy depends on the shape of the curve and, for proteins, the number of steps in the unfolding process. The number of intermediate steps in the unfolding process can be determined by taking the ratio of calorimetric enthalpy to Van't Hoff enthalpy. For a truly two-state process, the calorimetric and Van't Hoff enthalpy ratio would be 1; the values would be the same. If they are not equal, it could indicate intermediate unfolding steps, a non-two state process, or it could be the result of a normalized concentration that does not capture exactly the

amount of correctly folded protein. The complete data is shown in Table 1 for freshly prepared and stored samples with varying TCH content. Each concentration was prepared and scanned in triplicate (n=3). Fresh samples were scanned right after they were prepared and the stored samples were prepared, stored for five weeks, and diluted to appropriate concentrations just prior to scanning.

Table 1. Thermal unfolding temperature and enthalpy data for lysozyme in TCH

	Fresh (n=3)	Fresh (n=3)	Fresh (n=3)	Lysozyme in TCH Stored 5 weeks (n=3)	
	0.1M NaAcetate Buffer, pH 4	Aqueous TCH (72.5%H2O)	Aqueous TCH (47.3%H2O)	Dilute Sample, RH 33% (~92%H2O)	Dilute Sample, RH 23% (~92%H2O)
Tm (°C)	75.75 ±0.04(0.05%)	75.81 ±0.19(0.25%)	82.32 ±0.92(1.1%)	74.16 ±0.26 (0.35%)	74.81 ±0.33(0.44%)
DeltaH (kcal/mol)	116.8 ±4.1(3.5%)	101.0 ±4.5 (4.4%)	106.8 ±6.4 (6.0%)	152.6 ±26.3(17%)	175.5 ±18.0(10%)
DeltaHv (kcal/mol)	125.2 ±0.8(0.6%)	124.0 ±2.8 (2.2%)	131.6 ±12.4 (9.4%)	131.8 ±5.3 (4%)	137.4 ±0.8 (0.55%)
Concentration from A280 (mg/mL)	1.007 ±0.04(4.4%)	1.227 ±0.05(4.5%)	1.146 ±0.175(15%)	1.065 ±0.03 (3.0%)	0.922 ±0.04(4.8%)

Lysozyme typically has a two-step unfolding process and the smooth DSC endotherms do not suggest any intermediate steps, see Figure 9. The enthalpies obtained in the table above are comparable to literature values. The first study reports calorimetric enthalpies for lysozyme in Sodium Acetate ($NaC_2H_3O_2$) buffer ranging from 103 to 105 kcal/mol[2]. The second study reports slightly higher enthalpy values, 122 to 129 kcal/mol for lysozyme in NaH_2PO_4, with the ratio of calorimetric to Van't Hoff enthalpies close to 1[3]. A calorimetric enthalpy value lower than the Van't Hoff enthalpy could indicate that not all the protein is folded correctly or that the concentration has been overestimated. Enthalpy values higher than the Van't Hoff enthalpy could indicate that the concentration has been underestimated. Another potential cause of discrepancy between enthalpy values could be variation in the baseline. For this study, the concentration of stored samples was measured a few days after they had been diluted and scanned in the DSC. It is possible that some of the protein had degraded in that time and that the concentration measure by absorbance was lower than the concentration in solution during the DSC scans.

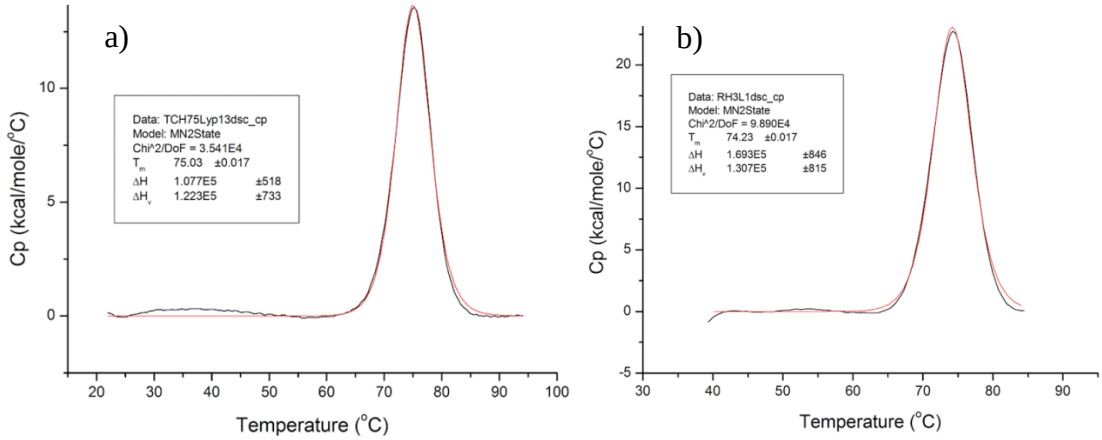

Figure 9. DSC thermal scans for a) Fresh Lysozyme in TCH with 72.5% H2O and b) Lysozyme after storage for 5 weeks in TCH solutions at controlled RH

3.3. Sorption Kinetics

Moisture sorption characteristics of the TCH composition were investigated as water content can have a significant effect on the stability of the solution. Samples were observed for crystal formation throughout these experiments. Figure 10 shows the moisture sorption kinetics for the TCH 1:4 molar ratio at room temperature for all of the relative humidity levels tested.

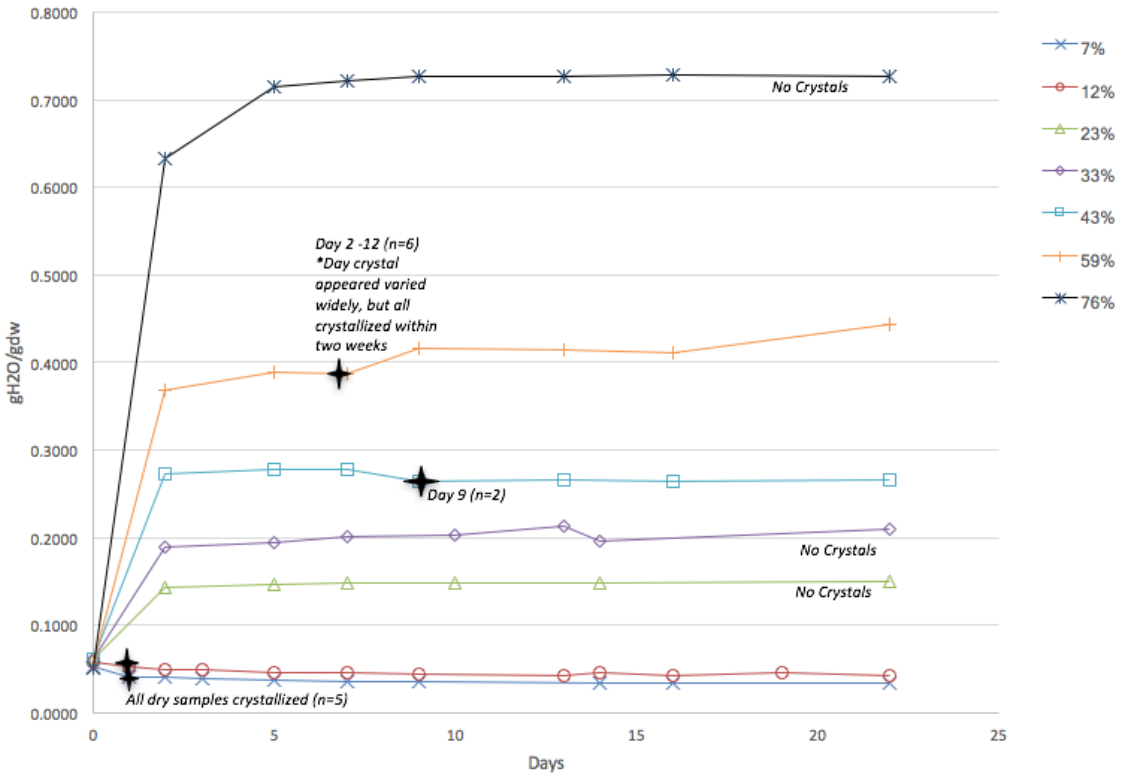

Figure 10. Sorption kinetics of trehalose-choline chloride 1:4 molar ratio at 20°C. The first day crystals were observed is marked with a star. n is the number of samples that crystalized on each day.

Samples picked up water quickly in the first few days and then gradually reached equilibrium within two to three weeks. Prior to storage, the dried samples with 0.02-0.08 grams of water per gram dry weight appeared cloudy and had a needle-like crystal structure under the microscope as shown in Figure 11. At relative humidity values below 23%, samples continued to lose moisture and remained cloudy.

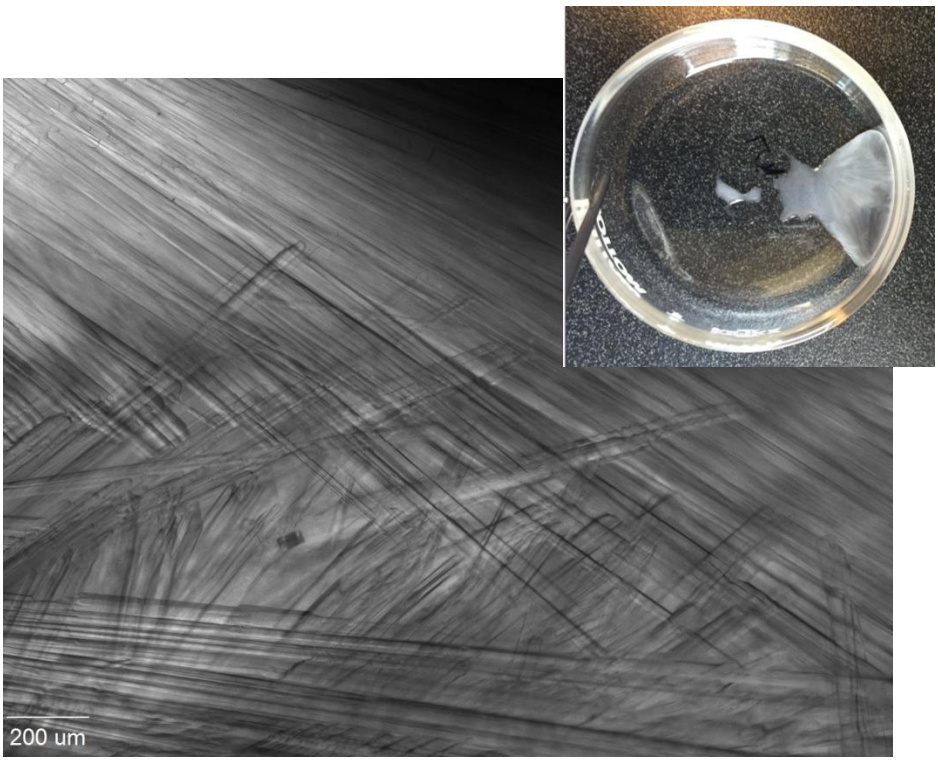

Figure 11. Dried TCH solutions, 4x magnification. Inset shows the sample without magnification

At relative humidity values of 23% RH and higher, all samples became clear within the first day or two and remained clear throughout the duration of this study. At RH of 43% and 59%, components started to precipitate out of solution. Samples at RH 75.5% appeared very water-like and only one of five replicates had components precipitate out of solution. The lack of crystals at the highest RH tested indicates that the solutions took on moisture quickly enough to bypass the supersaturated region and fully solubilize the sample. The crystal structures of the dry and supersaturated samples along with images of the liquid and highly dilute samples is shown in Figure 12.

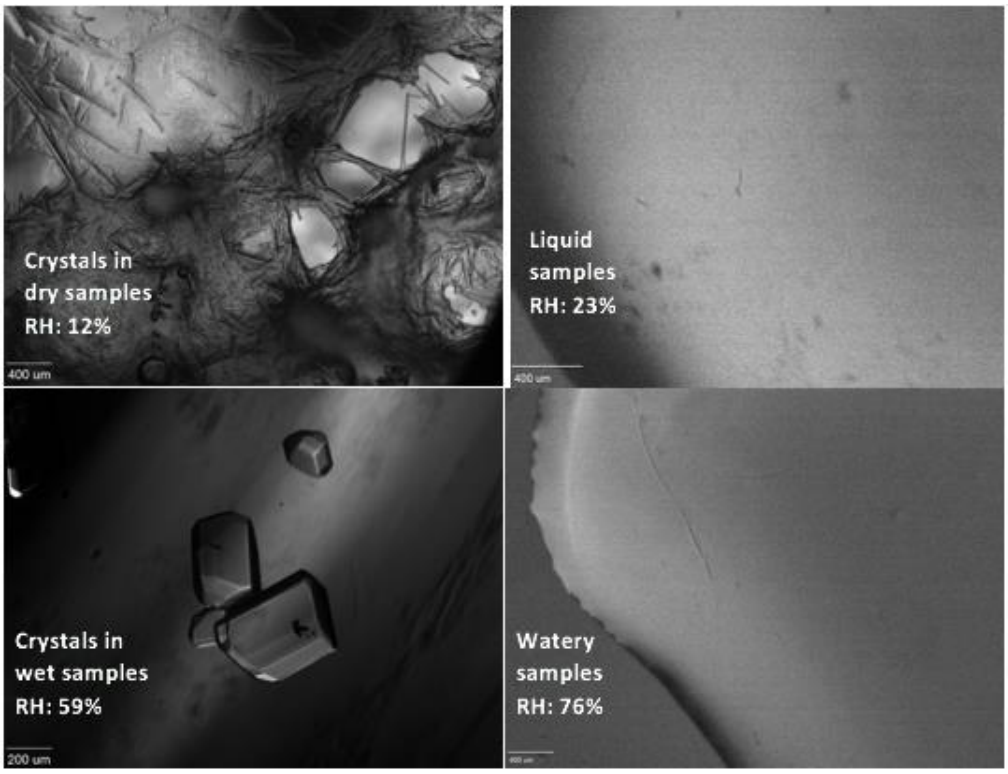

Figure 12. Samples after storage at the given RH. Crystals present in samples in low and high RH environments

As the dried samples were exposed to higher relative humidity values and began to take on moisture, the original needle-like crystals dissolved and the solution appeared amorphous. Samples became reflective and appeared wet. Figure 13 shows a dried sample that was sitting at room temperature exposed to the relative humidity of the environment for less than ten minutes. The crystal structure is barely visible.

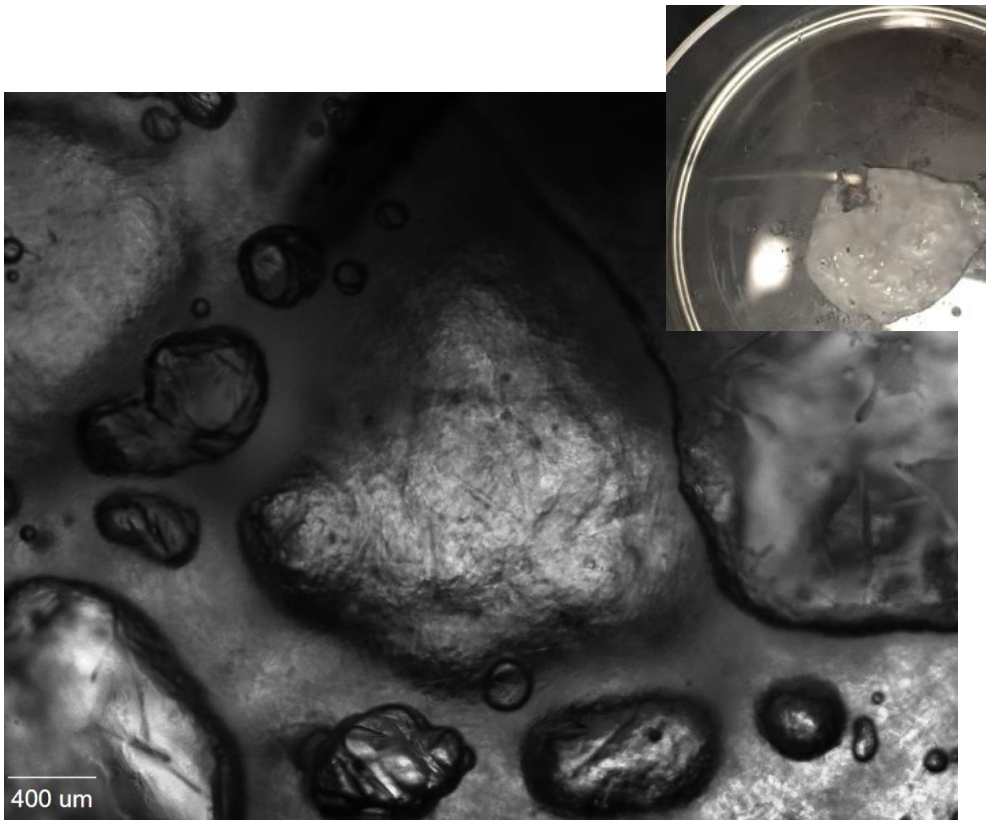

Figure 13. Evaporation dried TCH solutions, 2x magnification, as it begins to absorb moisture from the environment. Inset shows the sample without magnification

3.4. Moisture Sorption Isotherms

The sorption isotherm for amorphous trehalose is characterized as a Type 2, which is typical of water-soluble products[28, 30]. There is relatively low level of absorption at low humidity values consistent with monolayer adsorption, with a transition to multilayer absorption at higher humidity values. Shown in Figure 14 below are four trehalose sorption isotherms found in literature.

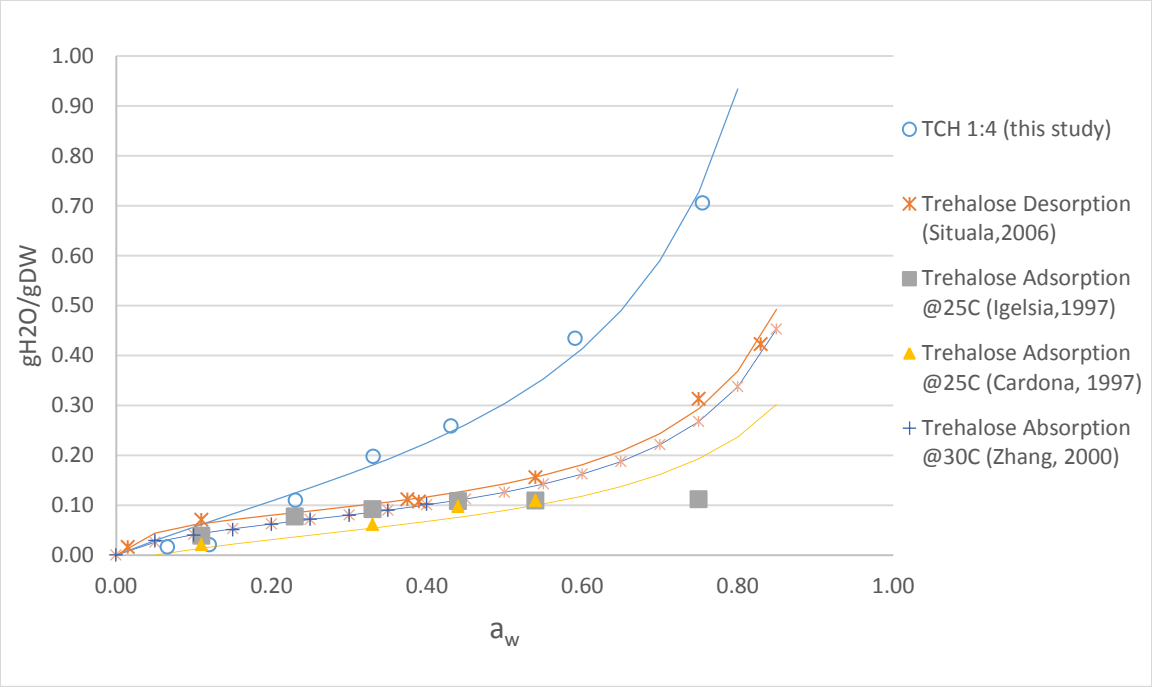

Figure 14. Moisture sorption isotherms for amorphous trehalose from the literature and from the results of this study [30, 35, 38-39]

As mentioned earlier, there can be significant differences between adsorption and desorption curves. For example, one study determined the desorption isotherm for a trehalose as a Type 2 isotherm[38]. The sorption isotherm reported by Iglesia, et al, would be characterized as a Type 1 isotherm where the amorphous sample takes on water up to a point and then forms the crystalline trehalose dihydrate[35]. Studies that begin with a crystalline sample of trehalose obtain isotherms that remain completely flat until the humidity value is close to the solubility limit for trehalose and the crystal dissolves into a solution[40].

The curves shown in Figure 14 were obtained in Origin Pro using the BET model. The fit parameters are shown in Table 2. The Type 1 isotherm reported by Iglesia, et al, could not be fit with the BET model.

$$\text{BET: } W(a_0) = \frac{W_B C_B a_0}{(1-a_0)(1+(C_B-1)a_0)} \quad (1.2)$$

Table 2. Model parameters for the BET equation obtained from Origin Pro

	Amorphous Trehalose, Desorption	Amorphous Trehalose, Adsorption	Amorphous Trehalose, Absorption	TCH
	Situala, 2006	Cardona, 1997	Zhang, 2000	This Study
W_B	0.07442	0.0642	0.06907	0.20237
C_B	24.59345	4.01077	10.46482	3.00417
r^2	0.9942	0.95167	0.9976	0.97809

Compared to the moisture sorption isotherms of anhydrous trehalose shown in Figure 14, the TCH composition absorbs water much more quickly. This is not surprising given that choline chloride is very hygroscopic, one would expect it to take on moisture more readily. The addition of choline chloride to the composition changed the isotherm from a Type 2 to a Type 3. The Type 3 isotherm, which is characterized by a steady increase in moisture content over the entire humidity range, indicates multilayer absorption without the initial monolayer formation of a Type 2 isotherm. The shift in the isotherm from a Type 2 to Type 3 also suggests that moisture is distributed evenly throughout the bulk of the material instead of accumulating on the surface first. The absorption process for a substance with a Type 3 isotherm is similar to the process of diffusion and correlates with a more stable liquid state.

During the sorption experiments, four regions were discerned. The delineations on Figure 15 are approximate as there were not enough activity levels tested to determine the exact location of property change. Dry samples with 0.03 or less grams of water per gram dry weight appeared cloudy and revealed needle-like crystals as shown previously.

There appeared to be a stable region where samples of TCH with water content between 0.1 and 0.2 grams water per gram dry weight stored at RH of 23 and 33%, respectively, showed no visible signs of crystallization for the duration of this study, over 60 days. Above this region was a metastable, supersaturated region that became liquid prior to crystals precipitating out of solution. The water content for these samples was around 0.4 grams water per gram dry weight (approximately 30 wt% water) which is below the solubility limit of pure trehalose, 53% water by weight[40].

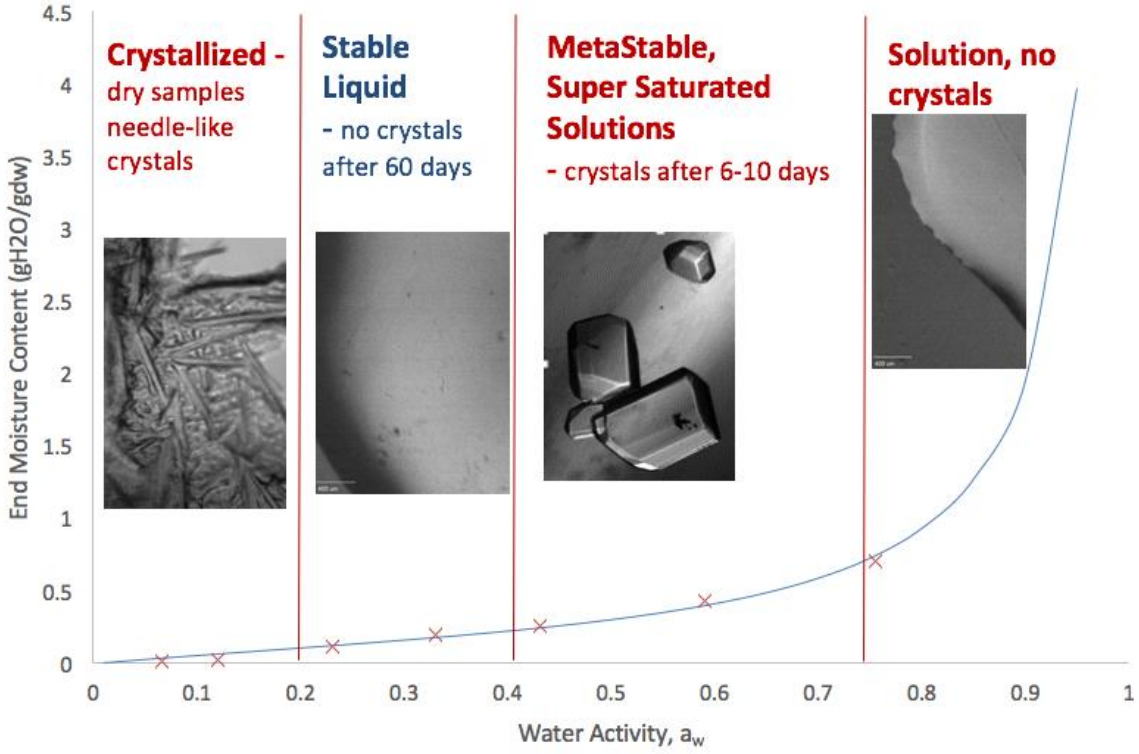

Figure 15. Moisture sorption isotherm for the TCH composition. End moisture content is reported as an average of five samples. The fit parameters are listed in Table 2.

The stable range of moisture content produced from this study corresponds to 9.8 to 16.5% water by weight with crystallization observed in samples with less than 5 % water or more than 20.6% water. The crystallization of components in solutions with

20.6% water suggests a lower stability limit than previous reported. The previous study reported the presence of additional hydrogen bonding for TCH compositions with 25% water by weight[21]. This could be the case, but the crystallization of components in samples with 20% water suggests that the hydrogen bonding is not enough at this moisture content to maintain the stability of the liquid. The previous study did not have any information for compositions with water content between 0 and 25%. This could be useful for determining a more precise range of stability

Crystals were visible to the naked eye, however, to present the structure more clearly a 4x magnification is shown in Figure 16. These crystals have a very similar structure to the crystals formed from a supersaturated solution of pure trehalose (see Figure 17). Supersaturated choline chloride forms many small crystals and the solution appears cloudy in contrast to the large, distinct trehalose crystals.

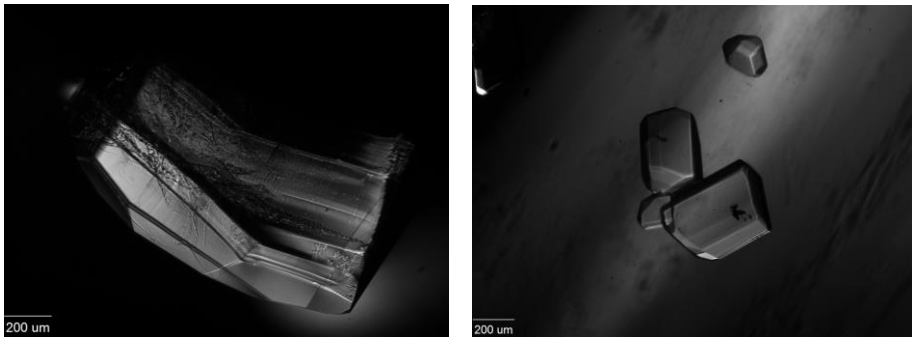

Figure 16. Crystals formed in TCH samples stored at RH 59%, 4x magnification

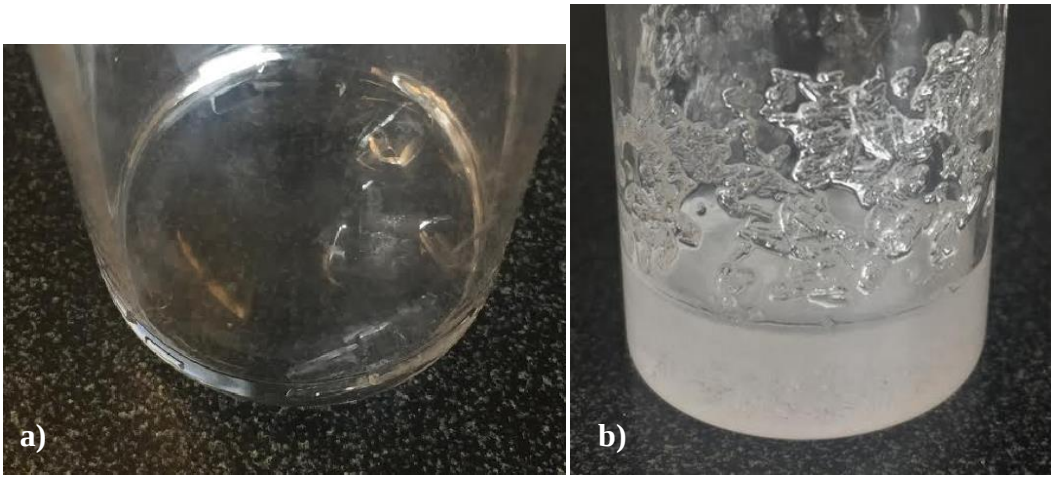

Figure 17. Crystals formed from a supersaturated solution of a) pure trehalose and b) choline chloride

Samples stored in very wet environments, RH of 75%, hydrated very quickly and did not form crystals. The water content of these solutions varied from 40 to 42% (grams water per gram solution). This indicates that the trehalose and choline chloride components are fully soluble at the moisture content maintained at this RH. The solubility limit for pure trehalose is reported to range from 42.3% (grams trehalose per gram solution) at 10°C to 59.7% at 40°C[40]. At 20°C, the solubility is reported as 46.6%, which corresponds to a water content of 53.4% (grams water per gram solution).

CHAPTER 4: CONCLUSIONS AND FURTHER STUDIES

From the results of this study, it is reasonable to conclude that there exists a range of water contents over which the trehalose choline chloride 1:4 composition remains stable without crystallization of components. When stored at relative humidity values of 23 and 33% (typical human comfort environmental conditions maintained in indoor environments), the water content of the solution reached an equilibrium of 0.15 and 0.21 grams water per gram dry weight, respectively. Crystallization did occur for samples with water contents below 0.05 and above 0.26 grams water per gram dry weight. The stable region suggests ideal moisture contents for utilization and storage of solutions and potential protein formulations. This information suggest that monitoring and controlling moisture content in these compositions is important for long term storage and stability.

Maintaining TCH compositions with a stable moisture content was another area considered in this study. Pure trehalose has a Type 2 isotherm, common for water-soluble products, where monolayer adsorption occurs on the surface up to the solubility limit and moisture then diffuses throughout the bulk of the sample. The sorption isotherm obtained for the TCH composition does not have the monolayer formation. TCH begins absorbing moisture throughout its bulk from the beginning and is characterized as a Type 3 isotherm. The tendency to absorb moisture throughout the bulk of the sample is similar to the process of diffusion and indicates a more stable liquid state. For stable liquid protein formulations, this is promising.

This composition also appears to be a good solvent for the model protein lysozyme. The protein was soluble in the TCH composition up to at least 40mg/mL at all water content levels tested (0.01 to 0.70 grams water per gram dry weight) and the protein showed no sign of degradation after five weeks of storage in stable solutions. Based on the results of this study, trehalose choline chloride compositions in ratios that lead to deep eutectic compositions may prove useful for liquid protein formulation.

Lysozyme has a long shelf life and is relatively stable compared to many therapeutic proteins. To better understand the role this composition may play in stabilizing proteins, future work might include longer storage studies with a thermally unstable or otherwise sensitive protein could be useful. Another suggestion for future would be to explore the effect of TCH formulation on protein activity before and after storage. Since protein function is highly dependent on its structure and preserving function is crucial for therapeutic formulations, measuring how well this composition preserves functionality for a given protein would be another important indicator of its potential as a novel protein solvent.

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