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Solid-State Stabilization of α-Chymotrypsin and Catalase with Carbohydrates

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We report a systematic investigation of the effects of five carbohydrates (fructose, sucrose, trehalose, maltotriose, and dextran), freeze-drying, and subsequent heat treatment (up to 8 days at 80 °C) on the structure and enzymatic activity of α-chymotrypsin and catalase. Importantly, we investigate high carbohydrate-to-protein mass ratio conditions (20:1 by mass) so as to minimize protein−protein interactions (e.g., protein aggregation). The activity of α-chymotrypsin is almost quantitatively recovered (greater than 80% of the initial activity) after lyophilization with or without added carbohydrates, and its conformation in the dried solid state as well as in an aqueous solution (after rehydration of lyophilized samples) is unchanged by the presence of carbohydrates. In contrast, catalase lyophilized without carbohydrates loses almost half of its activity upon reconstitution and shows significant structural changes in the dried state. The loss of activity and the lyophilization-induced structural alteration for catalase are appreciably reduced by addition of the lower molecular weight carbohydrates (fructose, sucrose, and trehalose). For protein preservation at elevated temperatures, the addition of carbohydrates at high carbohydrate-to-protein ratios (20:1 by mass) creates a glassy matrix dilute with respect to the protein, effectively minimizing intermolecular protein−protein interactions that could lead to protein aggregation in the solid state. It is also shown that the Maillard reaction between carbohydrates and proteins, which causes a significant loss of enzymatic activity, is successfully suppressed in glassy systems with less than 1% (w/w) water content. More significantly, a quantitative comparison is established for the first time between activity loss and the extent to which the Maillard reaction proceeds for dry glassy systems (as measured spectroscopically). Trehalose was found to be the most effective protector against prolonged heating: approximately 90% of α-chymotrypsin activity and 30% catalase activity are recovered upon reconstitution following 8 days at 80 °C in a trehalose matrix.

Introduction

One of the major challenges in the production of proteins as commercial pharmaceutical products is developing formulations that are stable during packaging, shipping, and long-term storage. Although liquid formulations are economical and convenient to manufacture, the transportation and long-term storage of labile proteins in aqueous solution are generally difficult because proteins in solution are susceptible to physical and chemical degradation (e.g., aggregation and hydrolysis).[1,2] These degradations, which may irreversibly damage the biomolecules, can occur even with a mild change in solution conditions, such as temperature or pH. Thus, proteins are often formulated as dried powders with additives that help to maintain their structural and functional integrity during formulation processing and storage. Such solid-state formulations constitute a stable pharmaceutical glass. Carbohydrates are one of the most important classes of pharmaceutical glass-formers for prolonging the shelf life of labile biological molecules.[3−7] The process of incorporating protein molecules into a glassy carbohydrate matrix involves extensive water removal from an initially dilute aqueous solution through a combination of freezing and drying (known as freeze-drying or lyophilization), resulting in a glassy state with water contents generally less than 5% (w/w).[8,9] The protein is therefore exposed to a wide range of temperatures, carbohydrate concentrations, and water activities before reaching the glassy state. The mechanisms by which carbohydrates stabilize proteins are likely to be quite different at the different stages in the process. Understanding these mechanisms and the specific interactions of proteins with the vicinal water and carbohydrate over such a broad range of conditions is a major scientific challenge and is also essential in developing a rational approach to the design of stable pharmaceutical formulations.

The detailed modes by which particular carbohydrates protect biological molecules remain incompletely understood. However, a number of interpretations have been proposed to explain their effectiveness as protein stabilizers under different conditions. It is generally accepted that preferential solute exclusion from the protein surface is responsible for the stabilizing effect of carbohydrates in aqueous solution.[10] This preferential exclusion phenomenon has been confirmed by experiments and computer simulations.[11−14] According to this mechanism, carbohydrates are preferentially excluded from the protein surface, thus increasing the protein's chemical potential. The degree of preferential exclusion and the protein's chemical potential increase with the amount of protein surface area exposed to the solvent. Since proteins in the denatured state have a comparatively larger surface area than in the native state, this leads to a correspondingly larger increase in chemical potential. As a result, the free energy difference between the native and denatured state is increased in the presence of carbohydrates, which thereby stabilizes the native protein in aqueous solution. This thermodynamic mechanism has also been invoked to explain protein stabilization by carbohydrates during freeze−thawing processes.[15]

During dehydration and storage, the protection of proteins appears to rely on the ability of carbohydrates to form glasses at low hydration levels.[16,17] In a glassy environment, character-

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ized by extremely high viscosity ($>10^{13}$ P) and sluggish molecular motion, most physical and chemical degradations, such as protein aggregation and deamidation, are expected to slow dramatically. However, it should be noted that although a low-moisture carbohydrate system is in the glassy state, a small molecule such as water is still able to retain significant mobility within the glass, as suggested by several experiments, as well as computer simulations.[18−24] It has been argued that the efficacy of some carbohydrates (such as disaccharides) to replace water and form hydrogen bonds with the protein is equally important.[25] By maintaining the hydrophobic driving force toward folding throughout the drying process, the formation of hydrogen bonds between carbohydrates and proteins would, in this interpretation, help to preserve protein structure and thereby enhance the activity recovery of the protein upon reconstitution.

Extensive experiments have been done to examine the merits of these interpretations.[26−38] The results of these experiments seem to indicate that vitrification is necessary but insufficient by itself for the stabilization of biomolecules. Therefore, fundamental understanding of the precise mechanisms by which carbohydrates stabilize proteins during freezing, drying, and long-term storage remains incomplete. In particular, the relative roles of kinetics and thermodynamics in solid-state stabilization have not been clearly elucidated. It is difficult to examine the effectiveness of different carbohydrates in stabilizing labile biological molecules under different conditions directly from these investigations because the model protein, water content, and sample preparation (e.g., buffers, lyophilization cycles, etc.) vary among the various studies. There is also much published work on the thermal stability of proteins in glassy carbohydrate matrices,[39−44] since preservation of biomolecules at elevated temperatures over an extended period of time is necessary in several practical applications. Examples include the potential application of carbohydrates in preservation of blood plasma proteins in either a liquid or solid state during viral inactivation at a typical temperature range of 60−100 °C[45−47] and the transportation of therapeutic products to areas with hot climates and poorly controlled storage conditions. However, since the effects of thermal stress on protein structure in glassy matrices and on protein structure after reconstitution, as opposed to just activity, were not investigated in these existing reports, the origin of activity loss after thermal treatment and its possible relation to the glassy state and the reactivity of carbohydrate/protein systems are still not fully understood. In addition, although the Maillard reaction between carbonyl groups (simple sugars) and amines (amino acids) leads to the formation of yellow-brown products[48] and is known to contribute to the chemical instability of solid proteins at elevated temperatures,[39,40,44,49] its effect on enzymatic activity recovery has not been studied in a quantitative manner.

The objective of this work is to improve our current understanding of the fundamental mechanisms of solid-state protein stabilization by carbohydrates. We are particularly interested in addressing two issues. First, we want to know how different carbohydrate properties, such as molecular weight and glass transition temperature (T_g), affect the efficacy of these molecules to preserve protein conformation and functionality during lyophilization and subsequent heat treatment. Second, we want to determine what role the carbohydrate plays in maintaining protein native structure in either a glassy state or aqueous solution (after rehydration of solid samples) in relation to its activity recovery upon reconstitution. As a step toward answering these questions, the present study systematically evaluates the effects of freeze-drying, storage temperature, and heating time on the structure (in the glassy state and after reconstitution) and enzymatic activity of two enzymes in the presence of a range of carbohydrates. The two model proteins are α-chymotrypsin and catalase, which differ greatly in their molecular weight and structure. α-Chymotrypsin is a relatively small enzyme that preferentially catalyzes the hydrolysis of peptide bonds involving L-isomers of tyrosine, phenylalanine, and tryptophan. It has a molecular weight of 25 000 Da. The α-helix and β-sheet contents of α-chymotrypsin determined by X-ray study are 9 and 34%, respectively.[50] Catalase is a large tetrameric enzyme with four identical subunits and has a total molecular weight of 250 000 Da. It is present in most animal cells and organs and in aerobic microorganisms. This enzyme protects these cells from the toxic effect of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water. Catalase has a far more complex structure and is intrinsically less stable. The free energy of unfolding ΔG_u for catalase is 41.9 kJ/mol[51] in comparison to 51.5 kJ/mol[52] for α-chymotrypsin. The X-ray analysis of its structure shows 29% α-helix and 14% β-sheet contents for this enzyme.[53]

The key features of this experimental study are summarized as follows. α-Chymotrypsin and catalase were subjected to identical lyophilization cycles and thermal treatments, accompanied by the same set of five carbohydrates with different molecular weights and glass transition temperatures. These carbohydrates are fructose, sucrose, trehalose, maltotriose, and dextran (MW = 68 800). Comparative analysis of the IR spectra of solution (no lyophilization), freeze-dried, and heat-treated samples provides detailed information on the effects of the different carbohydrates, freeze-drying, and subsequent heat treatment on protein secondary structure in glassy matrices, while analysis of the circular dichroism (CD) spectra of reconstituted samples gives valuable information on the reversibility, upon rehydration, of structural changes induced by freeze-drying and dry heating. The structural information obtained from the IR and CD spectra complements the data on recovered activity as a function of these same variables. A combination of these three sets of data thus provides significant insight into the structure−activity relationship in protein powders, as well as on the relative stabilizing efficacy of different carbohydrates during lyophilization and heat treatment. An identical set of experiments and analyses is also carried out for α-chymotrypsin and catalase in the absence of carbohydrates. Since the carbohydrate/protein formulations were prepared with a high carbohydrate-to-protein ratio (20:1 by mass), the comparative study of these carbohydrate/protein and pure protein formulations provides detailed insight into carbohydrate−protein and protein−protein interactions. The use of such a high carbohydrate−protein ratio is critical since it allows us to distinguish, by comparison with carbohydrate-free spectra, the signatures that are due to protein−protein interactions from those associated with protein−carbohydrate interactions. This helps us to understand and determine fundamental causes of protein instability in glassy systems, particularly at high temperatures. For instance, through this approach, we establish, for the first time, a quantitative comparison between activity loss and the extent to which the Maillard reaction proceeds for dry glassy systems (as measured spectroscopically).

This paper is organized in as follows: Materials and methods are described in section II, followed by results and discussion in section III. In section IV, we summarize the main findings and suggest possible directions for future work.

Materials and Methods

Materials. α-Chymotrypsin from bovine pancreas (C-4219), catalase from bovine liver (C-1345), and carbohydrates [fructose, sucrose, trehalose, maltotriose, and dextran (MW = 68 800)] were purchased from Sigma Chemical Co. (St. Louis, MO). α-Chymotrypsin and catalase were received as lyophilized powders. The buffers, salts, and all materials required for activity assays of α-chymotrypsin and catalase were also obtained from Sigma Chemical Co.

Preparation of Protein Formulations. Powdered α-chymotrypsin was used as received. It was dissolved in 10 mM Tris-Cl buffer (pH 7.0) to form a 5.0-mg/mL solution, which was then divided into six equal amounts. Unlike phosphate buffers, Tris buffers do not crystallize easily upon freezing, and the resulting pH shifts are thus minimized.[54,55] While one of the 5.0-mg/mL protein solutions was used as a pure protein control sample, one of the five different carbohydrates was added into each of the other five protein solutions, at a 20:1 carbohydrate-to-protein ratio by mass. Such a high carbohydrate-to-protein ratio creates a dilute matrix with respect to the protein concentration, and the results are thus not influenced by carbohydrate concentration-dependent effects. In addition, the high dilution minimizes protein aggregation. Catalase solutions were prepared in a similar manner, except that they were dialyzed prior to the addition of carbohydrates. The dialysis of catalase was carried out against 10 mM Tris-HCl buffer (pH 7.0) at 4 °C for 48 h. The final concentration of the catalase solution after dialysis was approximately 5.0 mg/mL, as determined by the absorbance at 280 nm. It is important to note that the concentration of protein solutions is high enough (e.g., 5.0 mg/mL) such that the recovered activity is not affected by small changes in the initial protein concentration of the aqueous solution.[56]

Protein samples were prepared by first dispensing 0.1 mL of protein or carbohydrate/protein solutions into 2.0-mL microcentrifuge tubes. The samples were quenched in a liquid nitrogen bath. They were then lyophilized with a VirTis AD-EL laboratory freeze-dryer using the following protocol: 15 h at −40 °C, 5 h at −20 °C, 5 h at 0 °C, and 2 days at 25 °C. The pressure was maintained below 30 mtorr throughout the entire drying process.[57,58] After completion of the freeze-drying, the samples were equilibrated over drierite for at least 2 days, and the tubes were then capped inside a glovebox under nitrogen purge. By visual inspection, the lyophilized formulations, except pure protein and fructose/protein samples, have a good solid cake structure (no structural collapse).

Moisture Determination and Heat Treatments. The water content of lyophilized samples was determined by thermogravimetric analysis (TGA) using a Perkin-Elmer TGA 7. A typical sample had a weight between 10 and 20 mg. During TGA measurements, a sample was first heated to 120 °C and then held at 120 °C for 30 min to ensure that its weight reached a steady value, indicating that the weight loss due to water evaporation was completed. All measurements were performed under a constant flow (30 mL/min) of dry nitrogen gas. The weight loss of a sample was recorded as a function of time and temperature. The final moisture contents of all lyophilized samples were found to be less than 1.0% (w/w). They agree with those measured with the Karl Fisher method using a Mitsubishi moisturemeter model CA-02 equipped with a Mitsubishi vaporizer model VA-02.

The lyophilized samples were heated to 60 or 80 °C, for 0.5, 1, 2, 4, or 8 days. Their water contents were kept lower than 1% (w/w) throughout the entire thermal treatments, since each sample tube was well sealed with an O-ring. These were confirmed by thermogravimetric analysis of these heat-treated samples.

Differential Scanning Calorimetry (DSC). Approximately 10−15 mg of lyophilized samples was placed in a stainless steel pan, which was immediately hermetically sealed with an O-ring to prevent any water gain. All DSC scans were performed using a Perkin-Elmer Pyris 1 differential scanning calorimeter equipped with a cooling accessory, Intracooler 2P. The DSC instrument was calibrated for temperature using both indium and cyclohexane and for enthalpy using indium. An empty sealed stainless steel pan was used as a reference. Lyophilized samples were first heated over a temperature range specific to each carbohydrate: fructose (−40 to 50 °C); sucrose (−10 to 100 °C); trehalose (−10 to 130 °C); maltotriose (−10 to 130 °C); dextran (−40 to 200 °C). This is henceforth referred to as the first DSC scan. The samples were then immediately cooled to their respective initial temperature and held isothermally for 30 min. After the completion of the isothermal hold, the samples were reheated to their respective end temperature, a procedure referred to henceforth as the second DSC scan. These heating and cooling scans were performed at a scan rate of 10 °C/min. All DSC scan results were normalized by the sample mass.

Enzymatic Activity Assays. Both α-chymotrypsin and catalase were assayed at 25 °C using procedures provided by Sigma. The activity assay for α-chymotrypsin is based on the ability of this enzyme to hydrolyze *N*-benzoyl-L-tyrosine ethyl ester (BTEE) to *N*-benzoyl-L-tyrosine.[59] The dried protein samples were first reconstituted with 1.0 mL of 10 mM Tris-HCl buffer per sample tube and then further diluted by up to a factor of 5, depending on the temperature and heating time of the heat treatment the sample was exposed to. The activity assay was initiated by mixing 0.10 mL of the diluted protein solution with 1.42 mL of 80 mM Tris-HCl buffer (pH 7.8), 1.40 mL of 1.18 mM BTEE, and 0.08 mL of 2 M $CaCl_2$ in a cuvette with a 1.0-cm path length. The increase in absorbance at 256 nm that results from the hydrolysis of BTEE was monitored for approximately 5 min. The initial linear slope of the absorbance vs time curve was multiplied by a dilution factor to determine the activity of α-chymotrypsin.

The activity assay of catalase measures the ability of the enzyme to decompose hydrogen peroxide to water and oxygen.[60] The dried protein samples were first rehydrated with 1.0 mL of 10 mM Tris-HCl buffer per sample tube and then diluted to a concentration that yielded a detectable activity level, ranging from 0.04 mg/mL for nonheated samples to 0.5 mg/mL for some samples that were heated for 8 days. A 0.1-mL volume of the diluted protein solution was added to a 1.0-cm path length cuvette that contained 2.9 mL of 0.036% (w/w) hydrogen peroxide. The time required for the absorbance at 240 nm to decrease from 0.45 to 0.40 was immediately recorded and then multiplied by a dilution factor to determine the activity of catalase.

In this study, the recovered activity (A) of both enzymes was expressed as a percentage of the activity in the control protein samples that were subjected neither to lyophilization nor heat treatment. The recovered activity of each formulation was averaged over three replicates for each sample. Activity assays of freshly prepared protein solutions with or without carbohydrates show equal activity (data are not shown here), confirming that the presence of the chosen carbohydrates did not affect the activity assays for α-chymotrypsin and catalase. Activity assays were also performed for pure α-chymotrypsin and catalase solutions with initial concentrations of 0.5−5 mg/mL to confirm

that these solutions have the same activity (normalized by the mass of proteins in solution) after they were stored at 4 °C for 4 h. This finding indicates that the protein solution remains stable at the concentration of 5 mg/mL prior to lyophilization.

Infrared (IR) Spectroscopy. IR spectroscopy was primarily used to probe changes in the protein secondary structure in the glassy state after lyophilization and heat treatment. IR spectra were recorded at room temperature using a Nicolet Magna 560 spectrometer equipped with a sample shuttle and a broad-band liquid nitrogen-cooled mercury cadmium telluride (MCT) detector. Each spectrum was generated by co-addition of 256 interferograms collected at a 4-cm^{-1} resolution, while the spectrometer was under a continuous dry air purge. For solid phase measurements, approximately 1.0 mg of each sample was first grounded with 300 mg of KBr. The dry mixture was then pressed into a pellet. This procedure for preparing pellets does not induce structural changes to dry solid proteins.[5] For liquid-phase measurements, aqueous solutions of native proteins (α-chymotrypsin and catalase) were prepared at a concentration of approximately 30 mg/mL. Spectra of these protein solutions were recorded immediately using an IR cell with BaF_2 windows and a 6-μm spacer, and the reference spectra were also recorded under identical scan conditions with only the corresponding buffer in the same cell. The protein spectra for aqueous samples were corrected according to previously established criteria for subtracting the contribution of liquid water.[61] If necessary, the spectrum of water vapor was subtracted from the solid or liquid sample.

The resolution of all IR spectra was enhanced by Fourier self-deconvolution (FSD)[62,63] to allow a better quantitative comparison and quantification of protein secondary structure by Gaussian curve-fitting in the amide I region (1700−1600 cm^{-1}). The full width at half-maximum (fwhm) and the enhancement factor (k) for FSD were set to 16 and 2.4, respectively. They are in the range of values used in the literature.[64−66] These FSD IR spectra were baseline-corrected and area-normalized to 1.0 for the amide I region. To further ensure that over-deconvolution was avoided, different combinations of fwhm (12, 16, 20, and 24 cm^{-1}) and k (1.2, 2.0, and 2.4) values were tested and the corresponding FSD IR spectra were analyzed by Gaussian curve-fitting. The quantitative estimation of secondary structural components (e.g., α-helix) was consistent among these parameter sets.

IR spectra were also analyzed by second derivatization in the amide I region. All second derivative spectra were smoothed with a nine-point Savitsky−Golay function, baseline-corrected, and normalized for area in the amide I region.[67,68] For each sample, the number of components and their peak positions were determined by the second derivative spectrum,[69,70] and they were also used as starting parameters for the Gaussian curve-fitting.

Circular Dichroism (CD) Spectroscopy. CD spectroscopy was used to investigate the secondary structure of reconstituted samples after lyophilization and heat treatment. CD measurements were made at room temperature with an Aviv 62 DS spectrometer. Aqueous solutions of native proteins and reconstituted samples were first diluted to 0.15 mg/mL and then placed in 2.0-mm path length quartz cells. CD spectra were recorded from 190 to 260 nm at 1.0-nm intervals. The CD spectra were corrected by subtracting the contribution of a Tris-HCl buffer from the original spectra and converted to the mean residue ellipticity (deg·cm^2/dmol).

Gel Electrophoresis. Native gel electrophoresis (in the absence of sodium dodecyl sulfate) was performed to qualitatively examine the effects of carbohydrates on the extent of dissociation of tetrameric catalase during lyophilization. The procedure is similar to the one used by Anchordoquy et al.[30,54] A 15-μL volume of a protein solution (no lyophilization) or a reconstituted lyophilized sample (1.5 mg/mL) was mixed with 10 μL of a buffer composed of 1 M Tris-Cl (pH 6.8), glycerol, and bromophenol blue (trace) before loading to a native 10% polyacryamide gel. A current of 35 mA per gel was applied, and the gel was run for approximately 2 h.

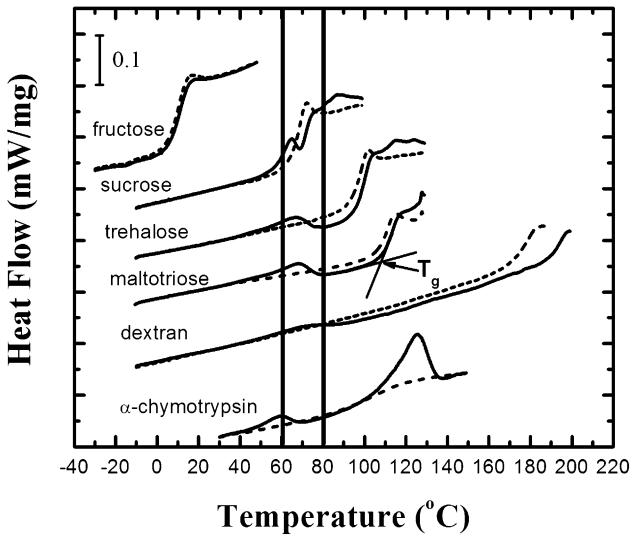

Figure 1. DSC scans of lyophilized α-chymotrypsin and carbohydrate/α-chymotrypsin (chy) formulations with water contents less than 1.0% (w/w). All DSC scans were performed at a scan rate of 10 °C/min. The first DSC scans are indicated by solid lines, and the second DSC scans (after 30 min of isothermal hold at the respective initial temperature) are indicated by dashed lines. The glass transition temperature (T_g) was taken as the onset of the heat flow increase in the first DSC scan (4 ± 2 °C for fructose/chy, 67 ± 3 °C for sucrose/chy, 94 ± 3 °C for trehalose /chy, 108 ± 2 °C for matlotriose/chy, and 184 ± 3 °C for dextran/chy). These T_g values are very similar to those of the corresponding carbohydrate/catalase formulations and pure carbohydrate glasses prepared using the same lyophilization cycles. Vertical lines indicate the two temperatures at which heat treatments were performed in this study.

UV Spectroscopy for Determination of Browning. After solid carbohydrate/protein formulations were heated, some of these formulations turned brown, which is a clear indication of the Maillard reaction or caramelization. UV spectroscopy was used to determine the degree of browning in these formulations by measuring the absorbance at 280 nm (I_{280}) in rehydrated samples. These aqueous samples were prepared by reconstituting solid samples with 10 mM Tris-Cl buffer and then diluted to 0.2 mg protein/mL with the same buffer. The browning intensity is defined in this work as a ratio of $(I_{280} - I_{0,280})/I_{0,280}$, where $I_{0,280}$ is the absorbance at 280 nm for the unheated solid sample.

Results and Discussion

DSC Analysis. Figure 1 shows DSC scans of lyophilized carbohydrate/α-chymotrypsin formulations and solid α-chymotrypsin. Since a high mass ratio of carbohydrate to protein was used in this study, the general features (e.g., glass transition temperature) of these DSC scans are very similar to those of the corresponding lyophilized carbohydrate/catalase formulations and pure carbohydrate glasses which underwent the same freeze-drying cycles (data are not shown here). The similarity of these three sets of DSC data suggests that the chymotrypsin and catalase are sufficiently dilute within the glassy carbohydrate matrix so that their presence does not measurably affect the T_g of formulations or carbohydrates. Despite some differences

Table 1. Secondary Structure Estimates for α-Chymotrypsin in Aqueous Solution (No Lyophilization), Lyophilized α-Chymotrypsin, and Carbohydrate/α-Chymotrypsin Formulations[a] and Recovered Activity of α-Chymotrypsin and Carbohydrate/α-Chymotrypsin Formulations after Lyophilization

formulation	intramolecular β-sheet (%)	α-helix (%)	random coil (%)	others[b] (%)	recovered activity[d] (%)
α-chymotrypsin (solution)	36	8	26	30	NA
α-chymotrypsin (solid)	30	15	14	41	95 ± 3
fructose/α-chymotrypsin (solid)	NA	NA	NA	NA	91 ± 5
sucrose/α-chymotrypsin (solid)	28	20	15	37	92 ± 4
trehalose/α-chymotrypsin (solid)	29	20	17	34	88 ± 3
maltotriose/ α-chymotrypsin (solid)	29	21	10	40	86 ± 4
dextran/α-chymotrypsin (solid)	30	10	15	45	81 ± 6
α-chymotrypsin[c]	34	9	57		NA

[a] Obtained by Gaussian curve-fitting in the resolution-enhanced amide I region. [b] Sum of turn, intermolecular β-sheet (for pure protein samples only), and undefined contents. [c] Obtained by X-ray analysis.[50] [d] Recovered activity is expressed as a percentage of the activity in control α-chymotrypsin samples that were neither lyophilized nor heated.

between the first and second DSC scans because of thermal prehistory effects, the glass transition temperatures determined from the first and the second DSC scans are similar. It should be noted that the second DSC scan is reproducible if the same sample is subjected to the identical DSC steps immediately after the second DSC scan. The average values of T_g for the carbohydrate/α-chymotrypsin formulations (from the first DSC scan) are given in the caption to Figure 1. They agree reasonably well with the literature values of T_g for dry carbohydrates.[71,72] It is important to note that fructose does not form a glass at room temperature and the T_g of sucrose is only slightly above 60 °C. Another key feature to note in Figure 1 is that no major thermal transition is observed for a dried α-chymotrypsin sample until it was heated to 100 °C, a temperature at which the thermal denaturation starts to occur as illustrated by a large endothermic maximum. This thermal denaturation temperature is much higher than that of α-chymotrypsin in solution, which is at approximately 55 °C, as determined by temperature-controlled CD measurements (results not shown). A small endothermic maximum, which is observed around 50 °C for the dried α-chymotrypsin sample, is simply due to the thermal prehistory effects and hence should not be associated with any significant thermal denaturation process. Such a small endothermic maximum has been previously reported for other dried proteins and nucleic acids.[73,74] A similar observation is also obtained for dried catalase (not shown here).

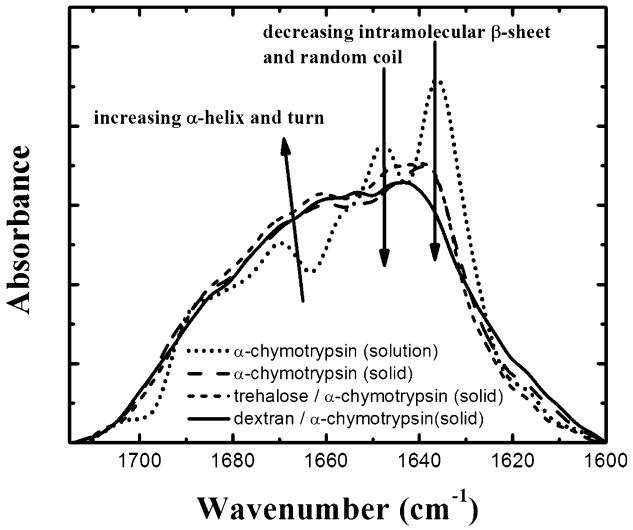

Figure 2. FSD IR spectra for α-chymotrypsin in aqueous solution (no lyophilization), lyophilized α-chymotrypsin, and carbohydrate/α-chymotrypsin formulations. The FSD IR spectra of sucrose/α-chymotrypsin and maltotriose/α-chymotrypsin formulations are not shown in this figure since they are similar to those of trehalose/α-chymotrypsin and pure α-chymotrypsin formulations, respectively. The arrows indicate the effects of freeze-drying on the secondary structure of α-chymotrypsin in the glassy state.

Protein Structure and Stability Analysis after Lyophilization. (A) α-Chymotrypsin. The FSD IR spectra in Figure 2 show the effect of carbohydrates (except fructose, which does not form a glass at room temperature) on the secondary structure of α-chymotrypsin in the glassy state after lyophilization. A comparison of the FSD IR spectra of α-chymotrypsin in aqueous solution (no lyophilization) and lyophilized formulations in Figure 2 indicates appreciable structural changes during lyophilization even in the presence of carbohydrates. The results of the quantitative analysis in the amide I region by Gaussian curve-fitting for these samples are shown in Table 1. These analyses show that α-chymotrypsin samples lyophilized with and without carbohydrates experience similar losses in their intramolecular β-sheet content (1636−1640 cm^{-1}), which is a major structural component of this enzyme. Consistent with the current literature,[67,68] the random coil content (1647−1648 cm^{-1}) is also found to be lower in the solid state (Table 1). The losses in these structural components are compensated by the increases in α-helix, turn, and intermolecular β-sheet structural motifs. The wavenumbers corresponding to α-helix for these lyophilized protein samples (1659−1662 cm^{-1}) obtained from the second derivative IR spectra are somewhat higher than the expected values (1655−1658 cm^{-1}) for regular α-helices. These small albeit significant shifts to higher wavenumbers can be attributed to the formation of shorter helices with some degree of irregular structure.[75,76]

Structural changes induced by lyophilization do not result in major activity loss for α-chymotrypsin upon reconstitution (Table 1). For instance, α-chymotrypsin samples that were subjected to freeze-drying with or without any carbohydrates showed at least 80% of their initial activity upon rehydration. This is confirmed by comparing the CD spectra of protein solution (no lyophilization) and reconstituted samples (including the fructose/α-chymotrypsin formulation). The CD spectra in Figure 3 show negative characteristic peaks at 203 and 230 nm, which are consistent with published data for α-chymotrypsin.[77] These CD spectra are different from those of typical β-sheet-dominated proteins, and this could be due to some irregularity of β-sheet structure in α-chymotrypsin.[78] It is nevertheless worth noting that the activity is actually degraded by the presence of carbohydrates. While this degradation is quite minor for fructose, sucrose, trehalose, and maltotriose, the dextran/α-chymotrypsin formulation shows a significantly lower activity recovery. This may be due to local phase separation, which cannot be detected by either IR or CD spectroscopy. Nevertheless, the similarity among the CD spectra shown in Figure 3 suggests that structural

Table 2. Secondary Structure Estimates for Catalase in Aqueous Solution (No Lyophilization), Lyophilized Catalase, and Carbohydrate/Catalase Formulations[a] and Recovered Activity of Catalase and Carbohydrate/Catalase Formulations after Lyophilization

formulations	α-helix (%)	intramolecular β-sheet (%)	others[b] (%)	recovered activity[d] (%)
catalase (solution)	33	20	47	NA
catalase (solid)	25	19	56	57 ± 3
fructose/α-chymotrypsin (solid)	NA	NA	NA	85 ± 7
sucrose/catalase (solid)	31	20	49	86 ± 7
trehalose/catalase (solid)	31	20	49	85 ± 6
maltotriose/catalase (solid)	26	20	54	60 ± 5
dextran/catalase (solid)	24	20	56	54 ± 5
catalase[c]	29	14	57	NA

[a] Obtained by Gaussian curve-fitting in the resolution-enhanced amide I region. [b] Sum of turn, extended chains, intermolecular β-sheet (for pure protein samples only), and undefined contents. The random coil content in all pure catalase and carbohydrate/catalase samples is negligible. [c] Obtained by X-ray analysis.[53] [d] Recovered activity is expressed as a percentage of the activity in control catalase samples that were neither lyophilized nor heated.

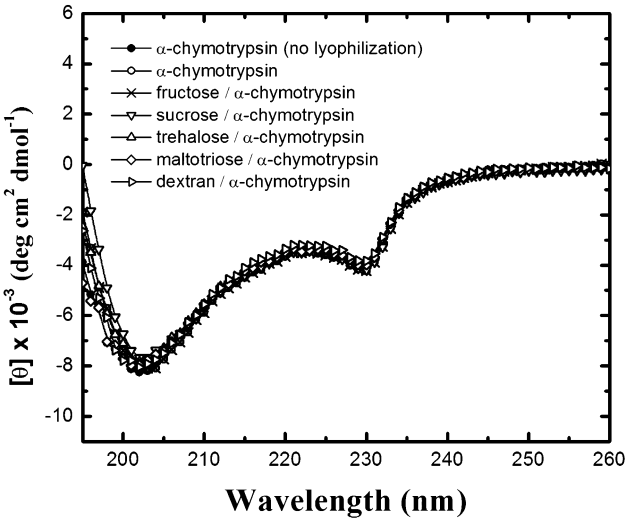

Figure 3. CD spectra of α-chymotrypsin in aqueous solution (no lyophilization) and lyophilized α-chymotrypsin and carbohydrate/α-chymotrypsin formulations after reconstitution.

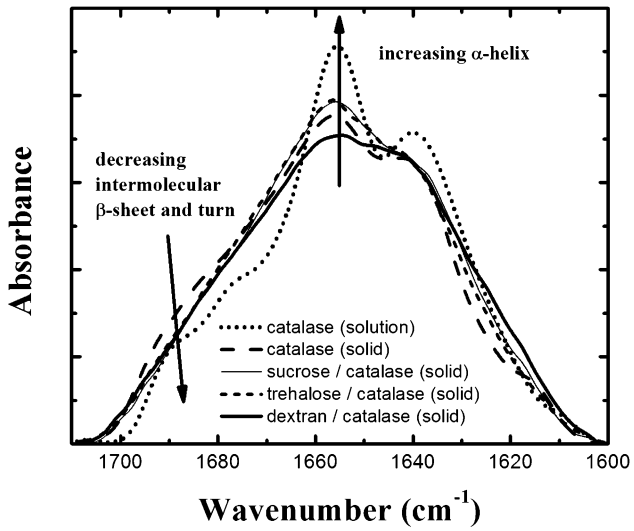

Figure 4. FSD IR spectra for catalase in aqueous solution (no lyophilization), lyophilized catalase, and carbohydrate/catalase formulations. The FSD IR spectrum of the maltotriose/catalase formulation is not shown in this figure since it is similar to that of the pure catalase formulation. The arrows indicate the effects of carbohydrates on the solid-state structure of catalase after lyophilization.

changes observed in the dried solids (Figure 2 and Table 1) after lyophilization are not significant in this case because they are reversible upon reconstitution.

(B) Catalase. The results are quite different for catalase (Figure 4 and Table 2). In comparison to the solution (no lyophilization) structure of catalase based on the IR data, the addition of disaccharides such as sucrose and trehalose to protein formulations helps to preserve the α-helix content (1656−1658 cm^{-1}) and reduce the formation of intermolecular β-sheet and turn structures (1680−1699 cm^{-1}) in the solid state after lyophilization, relative to the lyophilized catalase (no carbohydrate). It can be seen that the capacity of these carbohydrates to protect the protein native structure against freeze-drying stresses decreases noticeably as their molecular weight increases. Compared to the solid-state structure of catalase that is lyophilized without any carbohydrates, neither maltotriose (trisaccharide) nor dextran (polysaccharide) is effective in preserving the solid-state secondary structure. The results of the native gel electrophoresis (not shown here) are very similar for catalase in aqueous solution (no lyophilization) and lyophilized pure catalase and carbohydrate/catalase samples, showing a clear band near the 220 kDa marker. This suggests that carbohydrates have no major effect on the quaternary structure of this enzyme since no significant dissociation was observed even for the sample lyophilized in the absence of carbohydrates.

From the CD spectra (Figure 5), it can be seen that although the lyophilization-induced structural changes are not totally reversible upon reconstitution, the addition of small carbohydrate molecules (e.g., sucrose and trehalose) to catalase formulations

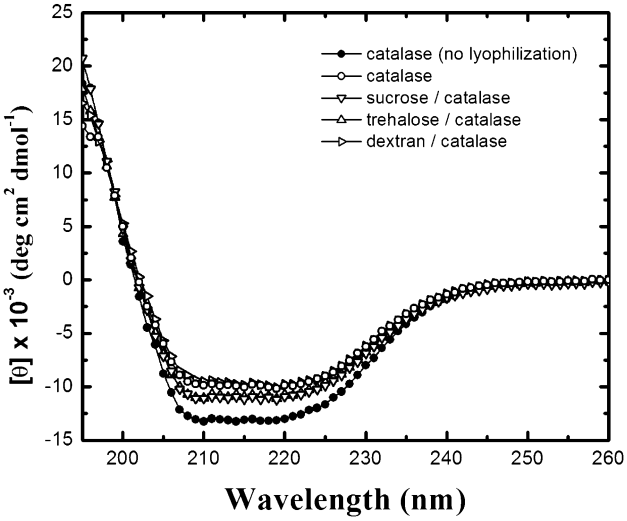

Figure 5. CD spectra of catalase in aqueous solution (no lyophilization) and lyophilized catalase and carbohydrate/catalase formulations after reconstitution.

during lyophilization leads to a higher retention of the native structure after rehydration. This is indicated by more negative ellipticity at 222 nm in Figure 5. This higher recovery of the native protein structure upon rehydration for disaccharide/catalase formulations is clearly related to the superior efficacy

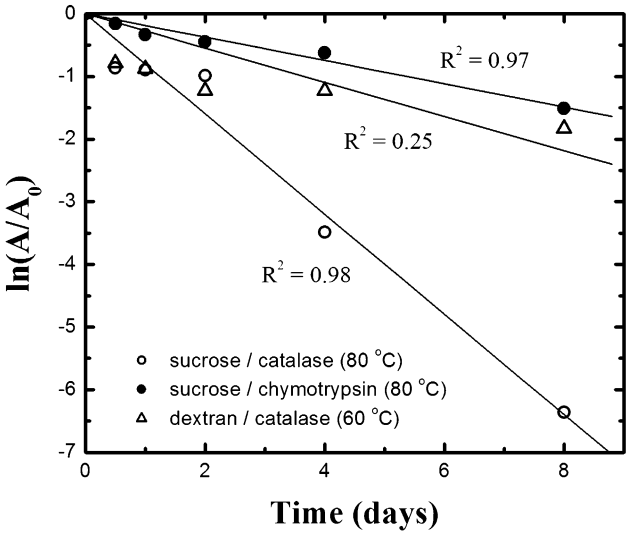

Figure 6. Representative plot of linear least-squares regression of degradation kinetics for catalase and α-chymotrypsin in sucrose at 80 °C and catalase in dextran at 60 °C.

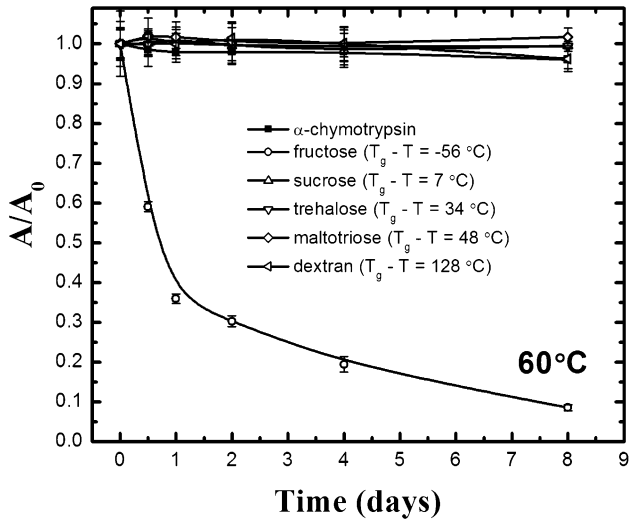

Figure 7. Recovered activity of pure α-chymotrypsin and carbohydrate/α-chymotrypsin formulations as a function of heating time at 60 °C. The recovered activity (*A*) is normalized by the activity of the unheated sample (A_0).

of disaccharides in maintaining the native protein structure in the glassy state after freeze-drying. Furthermore, the CD spectrum of the reconstituted fructose/catalase sample is identical to that of disaccharide/catalase formulations (this spectrum is not shown in Figure 5). This suggests that fructose is equally effective in preserving the native protein structure during lyophilization even though it does not form a glass during lyophilization.

The higher retention of the native protein structure in the glassy state and aqueous solution results in higher activity recovery of catalase upon reconstitution (Table 2). Fructose, sucrose, and trehalose are superior stabilizers against freeze-drying even though their glass transition temperatures are lower than those of larger carbohydrates, such as dextran.

The stabilizing ability of carbohydrates with respect to freeze-drying stresses depends not only on the physical properties of the carbohydrates but also on the nature of the protein. α-Chymotrypsin, the smaller enzyme, does not need carbohydrate stabilization when freeze-dried because it can refold to its native state and recover most of its activity after rehydration.

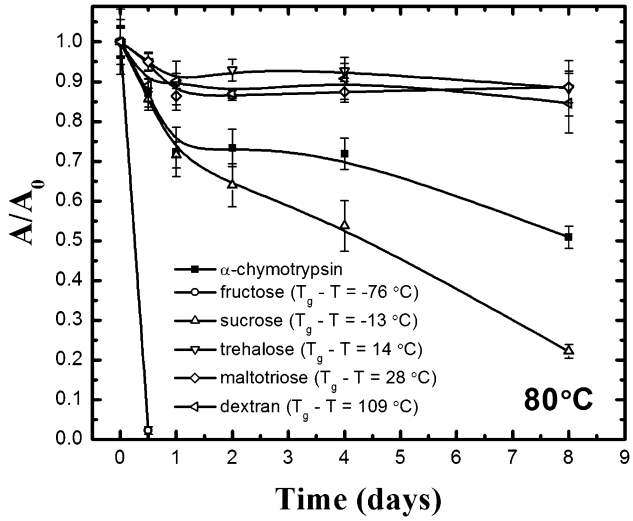

Figure 8. Recovered activity (see Figure 7 for definition) of pure α-chymotrypsin and carbohydrate/α-chymotrypsin formulations as a function of heating time at 80 °C.

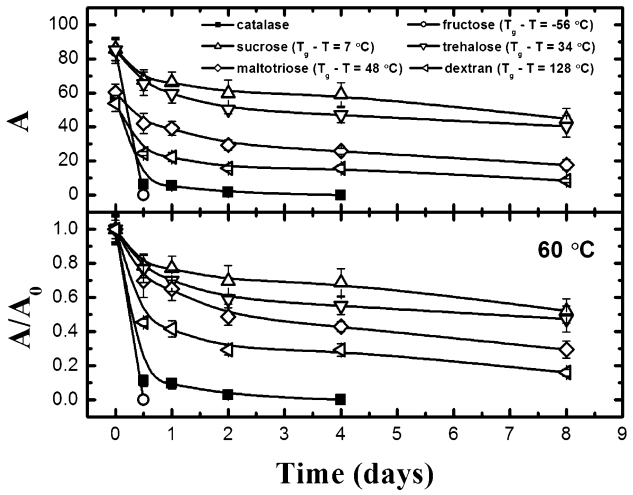

Figure 9. Recovered activity of pure catalase and carbohydrate/catalase formulations as a function of heating time at 60 °C. The un-normalized recovered activity (*A*) is also shown to indicate the marked differences in the initial activities among the lyophilized unheated samples (A_0).

In contrast, catalase shows a pronounced decrease in activity if it is not lyophilized with carbohydrates (fructose, sucrose, and trehalose). Both protein studies clearly indicate that the glassy state alone is not sufficient to account for protein stabilization during freeze-drying. In particular, the catalase results suggest that the extent by which particular carbohydrates interact with proteins (through hydrogen bonds, excluded volume, preferential exclusion, etc.) could play a significant role in determining the stabilizing efficacy of carbohydrates on protein conformation and enzymatic activity during lyophilization.[38, 79]

Kinetic analysis of the activity assay data was performed for both catalase and α-chymotrypsin. For each carbohydrate and heating temperature, the activity data were plotted according to various reaction orders and analyzed by linear least-squares regression. The best reaction order was determined based upon a comparison of the square of the correlation coefficients (R^2). First-order kinetics are obtained in the following cases: pure catalase (60 °C), catalase with sucrose (80 °C), and α-chymotrypsin with sucrose (80 °C), suggesting that degradation approaches or becomes first-order as the formulation is heated above its T_g. The R^2 values in these cases are at least 0.90, appreciably higher than the values obtained by assuming higher-

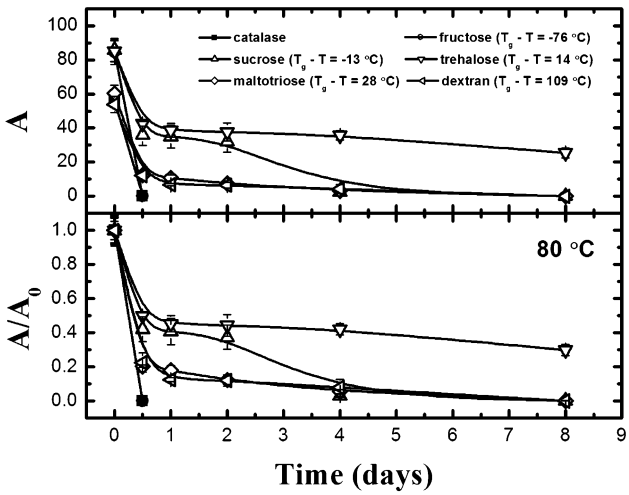

Figure 10. Recovered activity of pure catalase and carbohydrate/catalase formulations as a function of heating time at 80 °C. The un-normalized recovered activity (A) is also shown to indicate the marked differences in the initial activities among the lyophilized unheated samples (A_0).

order kinetics. For other cases, the best fits to the activity data give higher-order kinetics and do not lead to any meaningful conclusion. Figure 6 shows a sample plot of $\ln(A/A_0)$ vs heating time (first-order) for the sucrose/α-chymotrypsin and sucrose/catalase formulations heated to 80 °C and the dextran/catalase formulation heated to 60 °C. It can be seen that first-order kinetics provides a good representation of the data for both sucrose formulations but not for the dextran formulation.

Protein Structure and Stability Analysis after Heat Treatment. The relative ability of various carbohydrates to protect α-chymotrypsin and catalase upon heat treatment following lyophilization was also examined in this work. Dried solid formulations were heated to 60 or 80 °C and maintained at one or the other of these temperatures for period varying from 0.5 to 8 days. Figures 7 and 8 show a marked activity loss for fructose/α-chymotrypsin formulations after heating at both 60 and 80 °C and for sucrose/α-chymotrypsin formulations after heating at 80 °C. Lyophilized α-chymotrypsin samples (no carbohydrate) also experience pronounced activity losses over time at 80 °C, which is lower than the thermal denaturation temperature of dried α-chymotrypsin (100 °C). Carbohydrate/α-chymotrypsin formulations that have their T_g above these two heating temperatures show remarkable thermal stability. The activity loss in these carbohydrate/α-chymotrypsin samples is therefore a direct consequence of heating above the matrix T_g.

In contrast, catalase is more sensitive to thermal stresses, as illustrated in Figures 9 and 10. The pure solid catalase formulation shows a pronounced decline in activity after being heat-treated for half of a day at 60 °C. Catalase also loses all of its activity upon heating to 60 °C for half of a day in the presence of fructose (Figure 9). Other formulations experience a gradual but noticeable decrease in their activity after initial heating (2 days) although catalase is dispersed in the glassy carbohydrate matrix. Interestingly, the sucrose glassy matrix offers excellent protection to this enzyme despite that fact that the heating temperature (60 °C) is very close to its T_g. This indicates that the stability of catalase inside glassy carbohydrate matrices cannot simply be correlated with ($T_g - T$) below the glass transition temperature. The activity of both pure catalase and fructose/catalase formulations was abruptly reduced to zero after storage at 80 °C for half of a day (Figure 10). The sucrose/catalase formulation, when heated above its T_g, showed a sudden and significant activity loss after 2 days at 80 °C. The difference in the stabilizing effect of sucrose and trehalose on the enzymatic activity indicates that the glassy state is still critical in this case. Although encapsulation of catalase inside glassy carbohydrate matrices is important for minimizing undesired protein−protein interactions (e.g., protein aggregation) at 80 °C, only the catalase sample inside a trehalose matrix still exhibits significant activity after 8 days at this temperature. This may be closely linked to the ability of trehalose to preserve the activity of this enzyme after lyophilization (e.g., the trehalose/catalase formulation has significantly higher activity than the other formulations with T_g above 80 °C before heat treatment). This argument is consistent with the activity data for 60 °C (Figure 9), which shows the correspondence between activity preservation on lyophilization and on subsequent heating. For instance, the carbohydrate (e.g., maltotriose and dextran)/catalase formulations, which show poor activity recovery after freeze-drying, are also poor in retaining their activity upon subsequent heating at 60 °C. It is noteworthy that collapse (the matrix underwent dramatic shrinkage) and browning (the color of solid samples turned brown) were observed for carbohydrate/protein formulations that were heated above their T_g in both protein cases. The origin of the activity loss in these formulations as well as in pure protein samples after heat treatment will be discussed below.

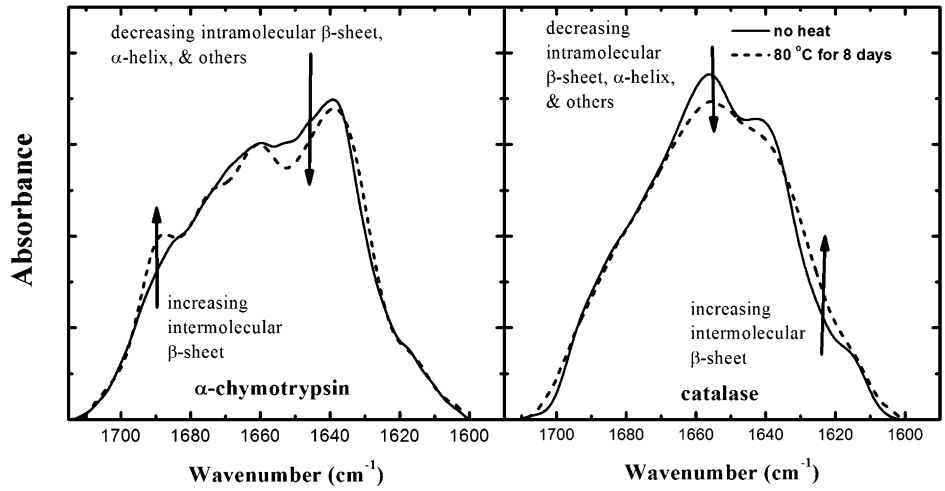

Figure 11. FSD IR spectra for pure α-chymotrypsin and catalase that were maintained at 80 °C for 8 days. FSD IR spectra of the lyophilized, unheated α-chymotrypsin and catalase samples are also shown for comparison. The arrows indicate the effects of heat treatment on the protein secondary structure in the glassy state.

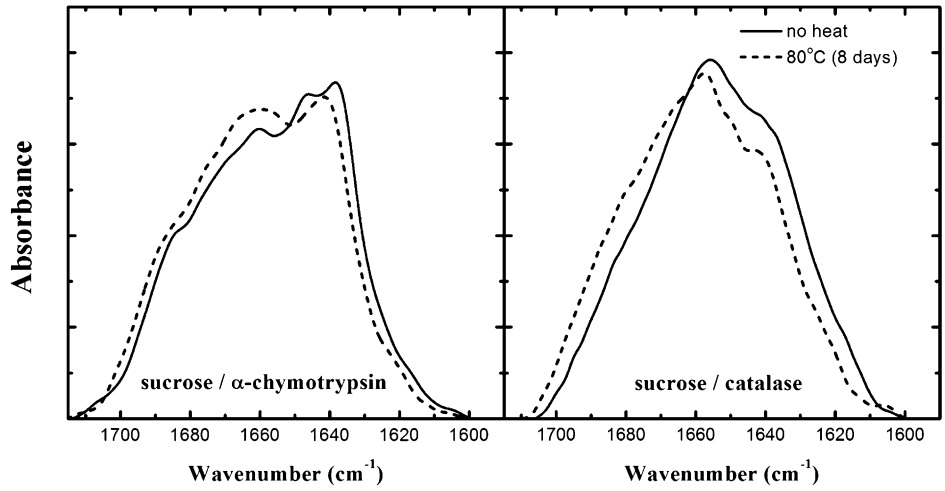

Figure 12. FSD IR spectra for α-chymotrypsin and catalase in sucrose matrices that were maintained at 80 °C for 8 days. FSD IR spectra of the lyophilized, unheated sucrose/α-chymotrypsin and sucrose/catalase samples are also shown for comparison.

To understand the molecular details associated with these activity losses, IR and CD spectroscopy were used to investigate the effects of temperature on protein secondary structure (α-chymotrypsin and catalase) in the glassy state as well as in aqueous solution after reconstitution. For solid proteins which were not lyophilized with carbohydrates, their FSD IR spectra (Figure 11), analyzed by Gaussian curve-fitting, show about a 4.0% increase in the intermolecular β-sheet component (1693 cm^{-1}) for α-chymotrypsin and a total of 5.0% increase in the intermolecular β-sheet (1625 cm^{-1}) and random coil (1650 cm^{-1}) components for catalase at the expense of the native structural motifs (intramolecular β-sheet, α-helix, etc.) after heating at 80 °C for 8 days. According to previous studies,[67,68] proteins in the solid state tend to aggregate at elevated temperatures. The resulting interactions with neighboring molecules give rise to intermolecular β-sheet formation but not to increases in disordered components. Such undesirable protein−protein interactions, however, can be effectively minimized by incorporating the biomolecules into a glassy carbohydrate matrix, which immobilizes the proteins and isolates them from each other.

After heating above T_g for an extended period of time, α-chymotrypsin and catalase within the sucrose matrix experience significant structural changes, as illustrated in their solid-state FSD IR spectra (Figure 12). The IR spectra for unheated protein/sucrose samples differ greatly from those for protein/sucrose samples that were exposed to 80 °C for 8 days. The origin of these structural disruptions is likely due to carbohydrate−protein interactions (the Maillard reaction), which will be discussed below. For solid carbohydrate/α-chymotrypsin formulations that were heat-treated below their T_g, the FSD IR spectra do not show any significant spectral changes within the amide I region and their corresponding second derivative IR spectra are also very similar (data are not shown here).

As illustrated in the CD spectra in Figures 13 and 14, the temperature-induced structural changes observed in the IR spectra of pure protein and sucrose/carbohydrate formulations are irreversible after rehydration. The CD spectra of fructose/protein formulations that do not form glasses at room temperature also show rather extensive temperature-induced structural perturbation after reconstitution. On the other hand, carbohydrate/protein formulations that were heated below their T_g (such as trehalose, maltotriose, and dextran formulations) show minimal changes from their corresponding native structure after rehydration in their CD spectra. It is interesting to note that these

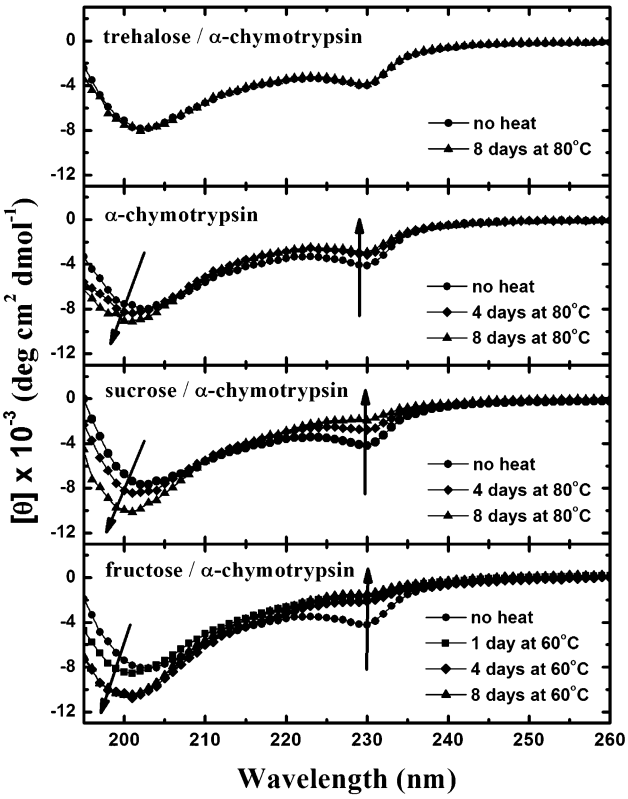

Figure 13. CD spectra for rehydrated trehalose/α-chymotrypsin, pure α-chymotrypsin, sucrose/α-chymotrypsin, and fructose/α-chymotrypsin samples after heating for various lengths of time at 60 and 80 °C. The arrows indicate the effects of heat treatment on protein secondary structure in aqueous solution.

spectral changes, indicated by the arrows in Figures 13 and 14, are similar to those that were observed during thermal denaturation of the corresponding protein in aqueous solution (data are not shown here).

Generally, there is a close correspondence between protein structure and enzymatic activity. Thus, it is insightful to compare the secondary structural data of heated samples after rehydration with their activity data. This comparison provides some fundamental understanding of the structural basis underlying enzymatic activity during protein stabilization by carbohydrates at elevated temperatures. For α-chymotrypsin, the activity loss is shown to be well correlated with the loss in the native

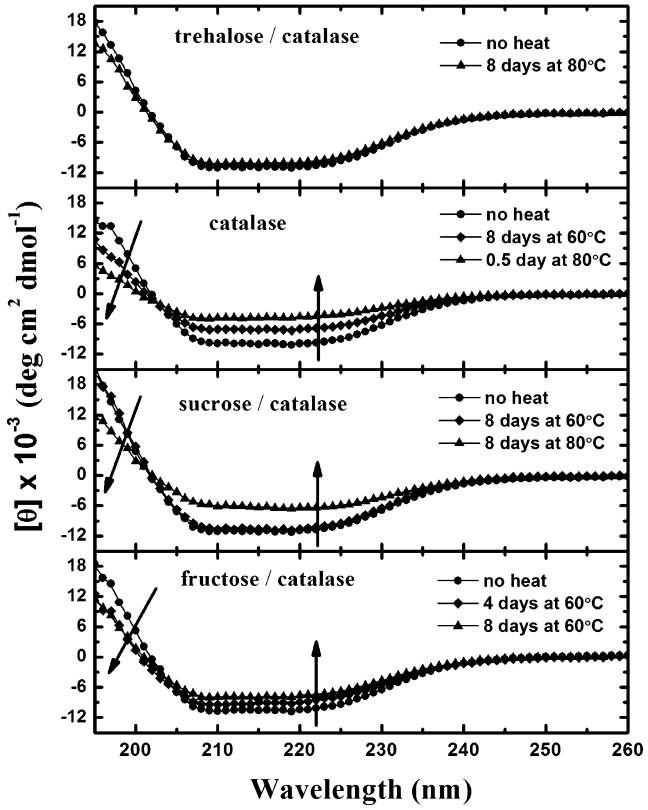

Figure 14. CD spectra for rehydrated trehalose/catalase, pure catalase, sucrose/catalase, and fructose/catalase samples after heating for various lengths of time at 60 and 80 °C. The arrows indicate the effects of heat treatment on protein secondary structure in aqueous solution.

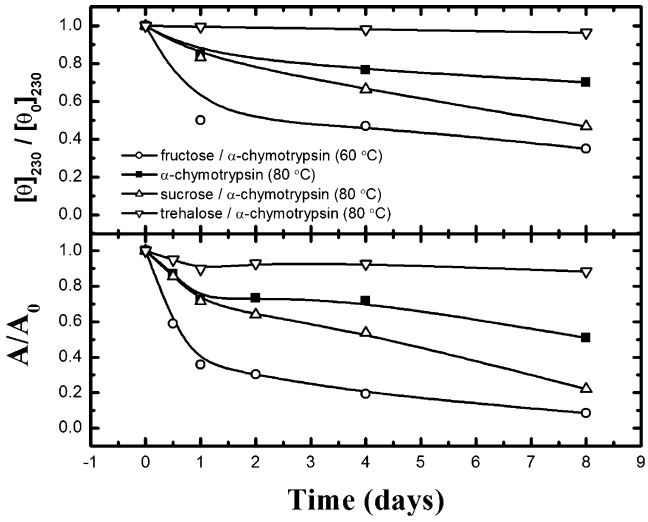

Figure 15. Comparison of structural effects (top) and recovered activity (bottom) after rehydration for α-chymotrypsin and carbohydrate (fructose, sucrose, and trehalose)/α-chymotrypsin samples exposed to different thermal treatments. $[\theta]_{230}$ and $[\theta_0]_{230}$ denote the ellipticity at 230 nm of the heated and unheated samples, respectively.

secondary structure, as indicated by the decrease in the negative ellipticity at 230 nm (Figure 15). This result illustrates that carbohydrates have similar stabilizing effects on the structure and biological activity of α-chymotrypsin at elevated temperatures. In contrast, pronounced inactivation of catalase can occur even without significant changes in structural features, as measured by changes in protein secondary structure (Figure 16). The structural alteration of this enzyme is indicated by the decrease in the negative ellipticity at 222 nm. One possible

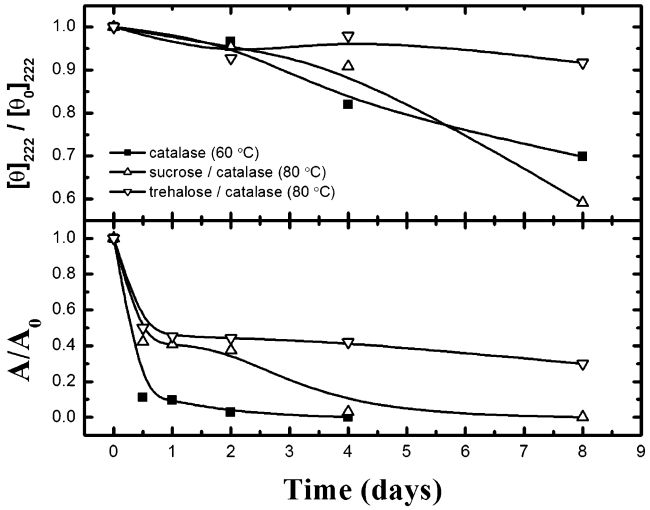

Figure 16. Comparison of structural effects (top) and recovered activity (bottom) after rehydration for for catalase and carbohydrate (fructose, sucrose, and trehalose)/catalase samples exposed to different thermal treatments. $[\theta]_{222}$ and $[\theta_0]_{222}$ denote the ellipticity at 222 nm of the heated and unheated samples, respectively.

microscopic picture consistent with this observation where enzyme inactivation occurs long before any significant structural changes has been suggested by Tsou.[80] In his hypothesis, the active sites within some proteins (e.g., tetrameric catalase), which are formed by relatively weak interactions, are more easily disrupted by thermal stresses than the enzyme molecule as a whole within glassy carbohydrate matrices. It should be noted that the activity loss may also be due to changes in the tertiary or quaternary structure.

Chemical Stability of Proteins in Carbohydrate Matrices. From the previous analysis of IR spectra, it is evident that the activity loss in pure solid protein samples during heat treatment is mainly caused by temperature-induced protein aggregation in the solid state. However, the activity loss in carbohydrate/protein formulations after severe heat treatment above the matrix T_g is primarily due to a chemical reaction between the protein and the carbohydrate, which is known as the Maillard reaction. Intensive browning and collapse of the glassy matrix were visually observed for carbohydrate/protein samples heated above T_g. Maillard products are usually complex but have a strong absorption in the ultraviolet region.[81] Thus, the extent of this browning reaction was quantified by measuring the absorbance at 280 nm in the reconstituted samples which had been subjected to heat treatment prior to rehydration. It is not surprising to see that the browning intensity of fructose/protein formulations increases dramatically with heating time (Figure 17) because these samples were heated significantly above their glass transition temperature (4 °C). In addition, fructose is a reducing sugar (the only reducing sugar studied here), this type of sugar being, in general, more Maillard-reactive.[82] In addition to its ability to react with amino groups through the Maillard reaction, fructose can undergo caramelization, a reaction in which the sugars react with themselves and produce an intense browning effect. Pure carbohydrate glasses that underwent the same lyophilization cycles were also subjected to the same heat treatment to examine the extent of caramelization in each carbohydrate. Among the carbohydrates used in this study, only fructose turned brown and showed a significant absorbance at 280 nm in the absence of proteins after heating at both 60 and 80 °C from 0.5 to 8 days (data are not shown here). Therefore, the increase in the browning intensity of fructose/protein formulations is due to both the caramel and Maillard reactions.

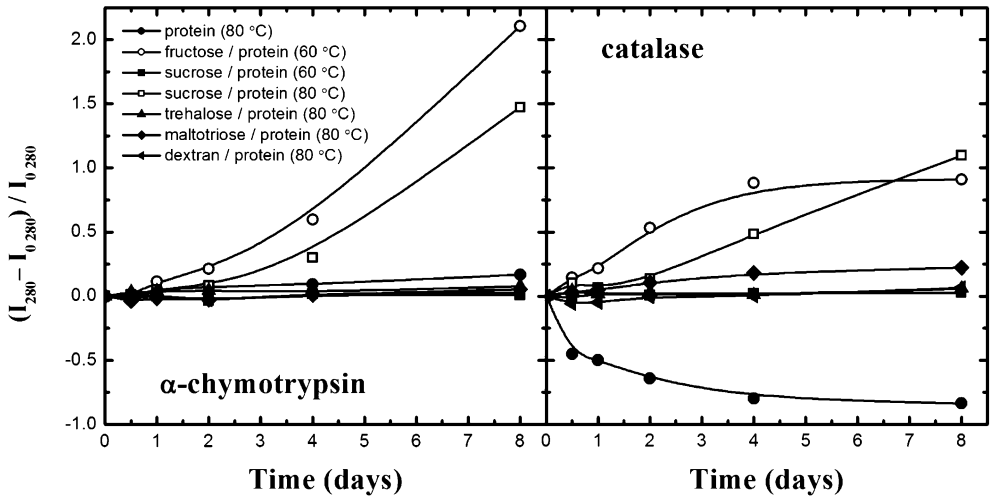

Figure 17. Browning intensity $[(I_{280} - I_{0,280})/I_{0,280}]$ as a function of heating time for pure protein and various carbohydrate/protein samples at 60 and 80 °C.

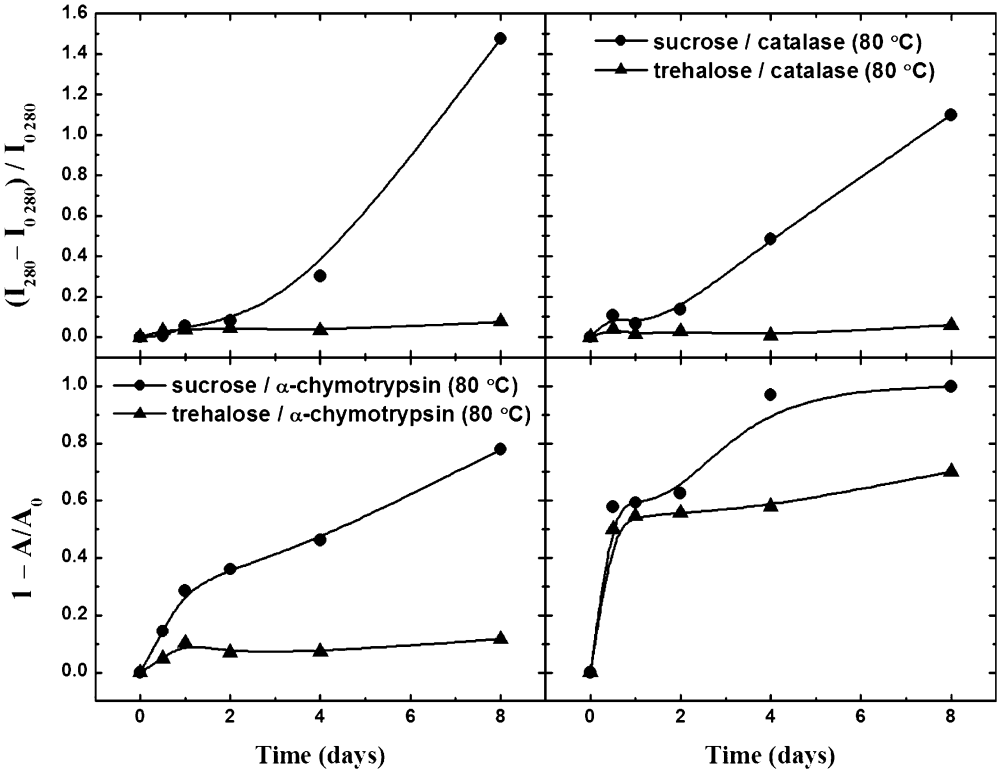

Figure 18. Comparison of browning intensity $[(I_{280} - I_{0,280})/I_{0,280}]$ with activity loss $(1 - A/A_0)$ at different heating times for sucrose/protein and trehalose/protein samples at 80 °C.

These reactions cause irreversible structural damage to both α-chymotrypsin and catalase in the presence of fructose (Figures 13 and 14), which also resulted in a significant activity loss upon reconstitution (Figures 7−10).

Although sucrose is a nonreducing sugar, sucrose/protein formulations also exhibited increased browning intensity after being heated to 80 °C (above the T_g of sucrose) for different periods of time, while other carbohydrate/protein formulations with T_g above 80 °C did not show any marked increase in browning intensity (Figure 17). Catalase's negative absorbance change with heating time in the absence of carbohydrates in this figure reveals that solid-state protein aggregation is severe for this enzyme. This is supported by the fact that the formation of large insoluble aggregates was observed in reconstituted catalase samples that went through extreme heat treatment. By plotting the activity loss $(1 - A/A_0)$ as a function of heating time for sucrose/protein and trehalose/protein formulations and comparing to the Maillard results (Figure 18), it was apparent that the additional activity loss in sucrose/protein formulations is strongly correlated with the extent to which the Maillard reaction proceeds (note in particular the additional activity loss in sucrose/catalase formulations with respect to trehalose/catalase formulations after heating at 80 °C for 2 days). This result indicates the importance of the glassy state in preventing the Maillard reaction in carbohydrate/protein formulations with less than 1% water content at high temperatures. One possible cause for the Maillard reaction to occur with a nonreducing sugar/protein formulation is that sucrose may be hydrolyzed to form two reducing sugars (glucose and fructose) during storage at 80 °C even at less than 1% water content. The decomposition

Table 3. Effects of Carbohydrates on Activity Recovery (AR) after Freeze-Drying and Heat Treatment[a]

	α-chymotrypsin			catalase		
carbohydrates (T_g) (MW)	freeze-drying (AR)	60 °C after 8 days (AR)	80 °C after 8 days (AR)	freeze-drying (AR)	60 °C after 8 days (AR)	80 °C after 8 days (AR)
fructose (4 °C) (180)	5	0[c]	0[c]	5	0[c]	0[c]
sucrose (67 °C) (342)	5	5	1[c]	5	2	0[c]
trehalose (94 °C) (342)	5	5	4	5	2	1
maltotriose (108 °C) (504)	5	5	4	3	1	0
dextran (184 °C) (68 800)	4	4	3	3	0	0
no sugar	5	5	2[b]	3[b]	0[b]	0[b]

[a] The activity recovery is expressed as a percentage of the activity in control protein samples that were neither lyophilized nor heated (5 = 100−85%, 4 = 84−75%, 3 = 74−50%, 2 = 49−30%, 1 = 29−10%, 0 = 9−0%). [b] Significant protein aggregation was observed. [c] The Maillard reaction was observed.

of a disaccharide to two monosaccharides becomes significant above T_g and thus increases the reactivity of the protein formulation considerably. Such decomposition has been observed previously even in the glassy system but with a much higher water content.[39] It has also been argued that the Maillard reaction is a diffusion-limited reaction.[83,84] A specific functional group of the protein and that of the carbohydrate must be close enough for the Maillard reaction to occur. Thus, it is difficult for the Maillard reaction to proceed in the glassy state because protein and carbohydrate molecules do not possess sufficient mobility.

Conclusions

We have presented the first systematic investigation of the ability of five carbohydrates (ranging from monosaccharides to a polysaccharide) to protect α-chymotrypsin and catalase during lyophilization and subsequent thermal treatment under high carbohydrate-to-protein ratio conditions (20:1). The effects of carbohydrates on protein stability as measured by activity recovery are summarized in Table 3 for different conditions. This table shows that there are appreciable differences in the stabilizing efficacies of these carbohydrates with respect to freeze-drying and thermal stresses. During lyophilization, α-chymotrypsin by itself demonstrates remarkable structural and functional stability. However, catalase, a tetrameric protein that is comformationally less stable, requires the protection of carbohydrates. In particular, it can be stabilized effectively only by fructose, sucrose, and trehalose, which are smaller in size and have lower glass transition temperatures than maltotriose and dextran. This implies that specific molecular interactions between the stabilizing compound and the protein are necessary, since vitrification by itself is not sufficient to confer protein stability during freeze-drying.

On the other hand, the stabilization of α-chymotrypsin and catalase by carbohydrates at elevated temperatures can generally be attributed to the elimination of undesirable intermolecular protein−protein interactions, as well as to the pronounced slowing of carbohydrate−protein interactions through dispersion of the biomolecules in a glassy matrix with a T_g higher than the temperature to which these enzymes were heated. At water contents of less than 1% (w/w), the glassy carbohydrate matrix by itself confers better thermal stability to these two proteins by preventing intermolecular protein aggregation and the Maillard reaction between the carbohydrate and the protein, provided they retain most of their biological activity after freeze-drying, as summarized in Table 3. Among the five carbohydrates studied here, trehalose demonstrates a superior ability to protect both proteins against stresses arising from both freeze-drying and heat treatment. The stabilizing efficacy of trehalose with respect to thermal stresses is related to its high T_g as well as its ability to preserve protein structure and activity upon lyophilization. Although there is noticeable deterioration of protein structural and functional stability as the temperature exceeds the glass transition temperature, for storage below T_g, protein stability does not appear to depend directly on the distance to the glass transition temperature ($T_g - T$).

The different behavior exhibited by α-chymotrypsin and catalase suggests that understanding how structural differences between proteins influence their stability is also important for elucidating the mechanisms of protein preservation by carbohydrates. Thus, it would be useful to extend this study by investigating systematically proteins that cover a wider range of molecular weights, amino acid sequences, and structural motifs. A careful investigation of the effects of moisture content on protein stability in glassy systems is also needed. Specifically, it would be important to study the effects of water content on the rate of the Maillard reaction in various glassy carbohydrate matrices, since previous studies have shown that this reaction can occur in the glassy state at higher hydration levels.[39] Although our thermal stability study suggests that kinetics play a key role in protein stabilization by carbohydrates at high temperatures (since $T < T_g$ is a necessary condition for stability), the role of kinetics and thermodynamics in protein stabilization during freeze-drying, particularly during dehydration, remain poorly understood. Knowledge in this area can be improved through the study of fructose−protein systems, which allows thermodynamic and kinetic measurements of protein stability over a wide range of water−carbohydrate compositions in the absence of a glass transition ($T_g \approx 4$ °C). Finally, we mention the issue of cooling rates. While our work did not address the relationship between cooling rates, solid-state protein structure, and activity recovery, this is an important process variable whose effect on product quality deserves careful attention.[55,58]

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