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Investigating Variables and Mechanisms that Influence Protein Integrity in Low Water Content Amorphous Carbohydrate Matrices

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Biopharmaceutical proteins are often formulated and freeze dried in agents that protect them from deleterious reactions that can compromise activity and authenticity. Although such approaches are widely used, a detailed understanding of the molecular mechanisms of protein stabilization in low water content amorphous glasses is lacking. Further, whilst deterioration chemistries are well described in dilute solution, relatively little is known about the extent and mechanisms by which protein integrity is compromised in the glassy state. Here we have investigated the relationship between protein modification and rate thereof, with variation of pH, carbohydrate excipient, temperature and the glass transition temperature using a model protein, lysozyme. Mass spectrometry analysis and peptide mapping confirm that protein modifications do occur in the glassy state in a time-, temperature-, and carbohydrate excipient-dependent manner. There were clear trends between the buffer pH and the primary modification detected (glycation). Most importantly, there were differences in the apparent reactivities of the lysine residues in the glass compared with those previously determined in solution, and therefore, the well-characterized solution reactivity of this reaction cannot be used to predict likely sites of modification in the glassy state. These findings have implications for (i) the selection and combinations of formulation components, particularly with regard to glycation in the glassy state, and (ii) the design of procedures and methodologies for the improvement of protein stability in the glassy state. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.*, 25: 1217–1227, 2009
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Introduction

In the *in vivo,* environment, proteins are sensitive to a number of well-characterized rearrangements, degradations, and chemical reactions that compromise the integrity of these proteins (e.g., the Maillard reaction, deamidation, and oxidation) and lead or contribute toward the progression of various disease states.[1] Animal species (in addition to others) have, therefore, developed protective mechanisms to either prevent or reverse these damaging reactions, or alternatively to remove and degrade such modified proteins. Such modifications are also observed in proteins *in vitro*, however, additional degradations and rearrangements are also often observed in solution including hydrolysis (particularly at Asp-Pro motifs), disulphide cross-linking and diketopiperazine formation,[2] all of which can result in the damaging loss of protein integrity, decreased or abolished bioactivity, and increased immunogenicity.[3,4]

There is, thus, an industrial requirement to prevent such modifications *in vitro* and preserve the integrity of proteins and peptides in both the food and biopharmaceutical sectors prior to their consumption or use to maintain biological authenticity. With particular regard to the biopharmaceutical industry, this is usually achieved via a range of strategies that involve formulating the protein of interest with protecting agents or excipients that protect the protein from deleterious reactions that might otherwise compromise their activity and authenticity.[5,6] Often these strategies have been optimized using empirical approaches.[7]

One standard practice/strategy utilized in both the food and biopharmaceutical fields is that of putting the protein/peptide of interest into the glassy state.[8,9] Practically this involves drying a peptide or protein, often with other glass-forming components, which are typically low molecular weight carbohydrates.[10] Drying is achieved by processes such as spray drying or freeze-drying. The mechanism by which this strategy prevents modification is thought to be the result of vitrification of the protein–carbohydrate mixture which, during freeze-drying, allows the formation of a readily dried open-pored cake[11] and

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subsequently, during storage, retards molecular motion and arrests chemical reaction on a practical timescale. A further application of the glassy state is protein stabilization during antiviral dry heat treatments. For example, freeze-dried Factor VIII is routinely treated at 80°C for 72 h, conditions that would result in rapid deterioration of the protein in the solution state, however, in the glassy state the protein is protected and virus inactivated. Indeed, we have previously shown that during antiviral dry heat treatment protein modification can occur to protein samples following freeze-drying.[12]

Although proteins dried in the absence of any additives form glasses,[13] those with added carbohydrate have been shown to have enhanced stability.[14] It has been proposed that the role of the carbohydrate is that of water replacement.[14] This is both in a general sense, that is, not only by filling the space which would be occupied by the water in the hydrated system, reducing the extent to which the secondary and tertiary structures of the protein are modified on drying, but also through a more specific interaction of the carbohydrate, particularly the disaccharide trehalose, with the protein which enhances stability.

Although there is a relatively large body of empirical work on how formulation and storage temperature affects the rates at which proteins in the glassy state either aggregate or lose their biological activity,[5] and there are several studies investigating such changes in the glassy state[15,16] our understanding of the molecular mechanisms of protein stabilization in glasses in the biotechnological setting is still far from complete. Further, while the deterioration chemistries are well described in dilute solution, relatively little is known about the extent and mechanisms by which protein (and peptide) integrity is maintained, or indeed compromised, by glassy state stabilization technologies.[17] Particularly at higher temperatures, protein unfolding may facilitate decomposition by new pathways, an area relevant to antiviral dry heat treatments of protein preparations. Although carbohydrate additives have been used to promote thermal stability of proteins, their molecular mechanism remains poorly understood and the potential to control the deterioration chemistry has not been exploited. Furthermore, a better understanding of the relationship between water content, temperature, formulation/carbohydrate excipient, and protein stability will help in the rational development of formulations that prevent or limit such potentially damaging reactions for the improved stability of food and biopharmaceutical products.[18,19]

The aim of this study was to address this gap in our knowledge using the model protein hen egg white lysozyme to investigate the relationship between chemical modification and rate thereof, pH, carbohydrate excipient, temperature, and the glass transition temperature. Mass spectrometry analysis and peptide mapping confirm that protein modifications do occur in the glassy state in a time-, temperature-, carbohydrate-, and pH-dependent manner. These findings have implications for (i) the selection and combinations of formulation components, and (ii) the design of procedures and methodologies for the improvement of protein stability in the glassy state to assure protein product quality.

Materials and Methods

Materials

All materials were of analytical reagent grade or better and purchased from Sigma Aldrich unless otherwise stated. Dextran (molecular weight 40,000) was sourced from Fluka. Commercial hen egg white lysozyme was sourced from Sigma and was of Grade 1 (provided three times recrystallized and lyophilized by the manufacturer).

Preparation of samples and formulations for freeze-drying

Protein formulations consisting of 5 mg/mL lysozyme were prepared with industrially relevant 3% sucrose, trehalose, and dextran excipients at pH 6.2, 7.2, or 8.2. To prepare the final solutions ready for freeze-drying, the required amount of excipient was dissolved separately in preprepared 0.1*M* sodium phosphate buffer pH 6.2, 7.2, or 8.2 containing 5 mg/mL lysozyme. The resulting protein solutions were then filtered, sterilized, and stored frozen (−20°C) prior to freeze-drying.

Freeze-drying of samples

Aliquots (1 mL) of the appropriate protein formulation (prepared as described above) were pipetted into 2 mL vials (Adelphi, Haywards Heath, UK) with a predried slotted stopper. The vials were then loaded onto a stainless steel tray and freeze-dried using a Virtis Advantage (EL 2.0) freeze-dryer (Biopharma Process Systems, Winchester, UK) using the following conditions: freeze the samples until shelf temperature reaches −45°C; hold the samples at this temperature for at least 60 min; primary dry at −35°C for 40 h; secondary dry at temperatures rising from −35 to −10°C at 100 mTorr over a period of 66 h before increasing the temperature to 20°C at 70 mTorr for a further 45 h. This protocol was developed based upon the glass transition behavior of a frozen sucrose-rich system. Often the freeze dryer would be loaded with samples with very different drying characteristics and so a slow drying protocol to accommodate all was used. Vials were then vaccuum dried in a vacuum oven over phosphorus pentoxide for 5 days. Finally, the vials containing the freeze-dried material were closed with a stopper and capped with aluminum caps. The amorphous nature of the samples was confirmed by the absence of crystals when viewed using polarizing light microscopy.

Determination of glass transition temperature and water content in freeze-dried samples

Glass transition temperatures (T_g) of freeze-dried samples were determined in triplicate after panning in a glove box under dry nitrogen conditions using a Perkin Elmer DSC 7 differential scanning calorimeter. An initial scan was performed to remove any effects of physical aging of the samples which can affect the apparent glass transition temperatures.[20,21] T_g's were determined on a rescan at a scanning rate of 10°C/min as described previously.[20,21] One exception to the use of this method was the sucrose pH 6.2 samples which were highly susceptible to buffer crystallization and so in this case the T_g was estimated from the first scan. A baseline was subtracted from the scan before analysis. The midpoint transition is quoted as determined by Wunderlich.[22] The water content of samples was determined in the differential scanning calorimeter (DSC) pans by puncturing the pan with a needle and then drying them to a constant weight (as determined using a Mettler ME30 balance) in a evacuated vacuum oven at 60°C over phosphorus pentoxide. After drying samples were cooled in a dessicator to room temperature before measurement.

Stability studies following freeze-drying of lysozyme in various excipient formulations

Following freeze-drying, all samples were stored in sealed vials at −80°C until analyzed or used for stability testing. For stability testing and accelerated storage studies the vials containing 1 mL aliquots of freeze-dried material were stored and incubated at either −80°C (control), ambient temperature (18–20°C), 37, 55, or 90°C for either 24 h, 1 month, or 16 months, prior to analysis as detailed later. Visual inspection of samples was undertaken immediately after storage and the details recorded. For more detailed molecular analysis, samples were reconstituted in the vial by reconstitution in 1 mL of ddH_2O.

Measurement of lysozyme activity

Lysozyme activity was determined by measuring the rate at which a known concentration of enzyme cleared a solution of the bacterial substrate *Micrococcus lysodeikticus*. Briefly, a 0.5 mg/mL suspension of *Micrococcus lysodeikticus* was prepared in PBS (pH 6.5) and stored on ice. This suspension was sonicated regularly and frequently to prevent aggregation or clumping of the cells. Reconstituted lysozyme solutions (20 μL) were then added to 1 mL of the substrate solution and the absorbance recorded every 10 s at 500 nm until a constant reading was obtained. All samples were analyzed in triplicate and the initial rate of clearance calculated to determine the lytic activity of the lysozyme sample in absorbance units/sec.

Electrospray ionization mass spectrometry analysis of lysozyme following freeze-drying and stability testing

Mass spectra were recorded in the positive ion mode on a Finnigan MAT LCQ ion trap mass spectrometer and the charge state ion distributions observed in ESI mass spectra deconvoluted as previously described[12] except that deconvolution was achieved using Excalibur software (version 1.2). Excipients were removed by reverse-phase high-performance liquid chromatography (HPLC) using a C_{18} reverse-phase column (250 × 2.0 mm^2 i.d., Phenomenex Jupiter, Macclesfield, Cheshire, UK) linked to an Agilent 1100 LC system before the sample was introduced into the mass spectrometer as previously described.[12]

Tryptic peptide mapping

Reconstituted lysozyme samples were reduced and alkylated with iodoacetic acid essentially as described by Smales *et al*.[12] Samples were dialyzed against 8*M* urea, 0.25*M* Tris-HCl buffer (pH 8.75) containing 1 m*M* EDTA and then 2.16 μmol dithiothreitol in 100 μL of the same buffer was added per 2 mg of lysozyme. The resulting solution was then left to incubate at 37°C for 3 h prior to the addition of iodoacetic acid (3 μL of a 2*M* solution per 2 mg of lysozyme). Alkylation was then allowed to proceed in the dark at room temperature for 45 min. β-mercaptoethanol was finally added (1% v/v) and the alkylated protein dialyzed against 8*M* urea. After dialysis, the alkylated protein solutions were diluted with 1% ammonium bicarbonate buffer to give 2*M* urea solutions. TPCK treated trypsin solution (4*M* in H_2O, 0.05% TFA) was then added to the digest solution so that an enzyme:substrate ratio of 1.5:100 (w/w) was achieved. The mixture was then left to digest for 5.5 h at 37°C before being stored at −20°C until required for further analysis.

Separation of the resulting tryptic fragments was carried out on a C_{18} reverse-phase column (Phenomenex Jupiter, 250 × 2.0 mm^2 i.d.) using an Agilent 1100 LC system. Aliquots from tryptic digests were loaded onto the column preequilibrated with deionized water (containing 0.05% TFA) and then eluted using a gradient from 0 to 70% acetonitrile (containing 0.045% TFA) in 100 min at a flow rate of 0.2 mL/min. Upon elution the peptides were introduced into a Finnigan MAT LCQ ion trap mass spectrometer and mass spectra recorded in the positive ion mode. The instrument was also setup to collect ms/ms spectra of all peptide peaks so that sequence information could be obtained in addition to peptide mass data. The charge-state ion distributions observed in electrospray ionization mass spectra were analyzed using Excalibur software (version 1.2).

Results

The physical appearance of lysozyme samples changes after freeze-drying and storage

Lysozyme is a protein that is readily obtained in large amounts, relatively small (14,306 Da) and thus highly amenable to analysis by mass spectrometry, and possesses an enzymatic activity that can be quickly, easily and cheaply determined. Further, this small enzyme has been extensively characterized and would therefore appear to be an ideal model protein with which to investigate a wide range of formulation and storage conditions as described in this work.

The physical appearance of the protein samples after freeze-drying was a white cake tightly filling approximately 10 mm at the bottom of the vial. The dextran samples appeared more finely textured than the sucrose and trehalose formulated samples (Table 1). Following storage at all times and temperatures investigated in this study, the dextran samples, irrespective of pH, exhibited little difference in appearance apart from a slight shrinkage in the volume (Table 1). With longer and higher temperature storage conditions however, the dextran samples took longer to reconstitute after addition of 1 mL of water (data not shown). The time taken for complete reconstitution was as long as 15 min which would be unacceptable in the medical setting. The trehalose samples also showed very little change in appearance after storage, the exception being the samples formulated at pH 6.2 and stored for 24 h at 90°C. This combination of carbohydrate and temperature exhibited marked shrinkage as compared with other trehalose samples; however there was no change in the ease with which these samples were reconstituted. In contrast, sucrose formulated samples showed more extreme change in appearance (Table 1). This was particularly noticeable for the sample formulated at pH 6.2 and stored at elevated temperatures. The sample stored at 90°C for 24 h was a hard brown mass at the bottom of the vial and was extremely difficult to redissolve. The sucrose samples formulated at pH 7.2 or pH 8.2 did not exhibit large changes in physical appearance and reconstituted with ease.

Glass transition temperatures (T_g) and water content following freeze-drying

Typical DSC scans showing heat flow per unit mass as a function of temperature are shown in Figure 1. All the samples with the exception of the sucrose pH 6.2 samples were

Table 1. The Appearance and Initial Lytic of the Freeze-Dried Lysozyme Samples After Incubation

		Incubation			Initial Rate (Δ OD/sec)	
Excipient	pH	Time	Temp	Appearance	Mean	SD
Sucrose	6.2	control	−80°	White, lacy	0.067	0.004
	7.2	control	−80°	White, slightly shrunken	0.078	0.002
	8.2	control	−80°	White, slightly shrunken	0.076	0.002
Trehalose	6.2	control	−80°	White, slightly shrunken	0.075	0.002
	8.2	control	−80°	White, slightly shrunken	0.077	0.003
Dextran	7.2	control	−80°	White, slightly shrunken	0.084	0.001
	8.2	control	−80°	White, slightly shrunken	0.093	0.001
Sucrose	6.2	24 h	90°C	Brown, small, hard	0.055	0.013
	7.2	24 h	90°C	White, small	0.105	0.004
	8.2	24 h	90°C	White, shrunken	0.089	0.008
Trehalose	6.2	24 h	90°C	White, shrunken	0.074	0.015
	7.2	24 h	90°C	White, slightly shrunken	0.115	0.003
	8.2	24 h	90°C	White, slightly shrunken	0.102	0.000
Dextran	6.2	24 h	90°C	White, slightly shrunken	0.068	0.001
	7.2	24 h	90°C	White, slightly shrunken	0.086	0.001
	8.2	24 h	90°C	White, slightly shrunken	0.072	0.009
Sucrose	6.2	1 month	55°C	Brown, small, hard	0.072	0.001
	7.2	1 month	55°C	White, slightly shrunken	0.076	0.001
	8.2	1 month	55°C	White, slightly shrunken	0.076	0.001
Trehalose	6.2	1 month	55°C	White, slightly shrunken	0.073	0.001
	7.2	1 month	55°C	White, slightly shrunken	0.075	0.001
	8.2	1 month	55°C	White, slightly shrunken	0.078	0.001
Dextran	6.2	1 month	55°C	White, slightly shrunken	0.075	0.002
	7.2	1 month	55°C	White, slightly shrunken	0.085	0.002
	8.2	1 month	55°C	White, slightly shrunken	0.078	0.002
Sucrose	6.2	16 month	37°C	Off white, small	0.094	0.003
	7.2	16 month	37°C	White, slightly shrunken	0.097	0.001
	8.2	16 month	37°C	White, slightly shrunken	0.091	0.006
Trehalose	6.2	16 month	37°C	White, slightly shrunken	0.074	0.003
	7.2	16 month	37°C	White, slightly shrunken	0.075	0.004
	8.2	16 month	37°C	White, slightly shrunken	0.072	0.002
Dextran	6.2	16 month	37°C	White, slightly shrunken	0.071	0.007
	7.2	16 month	37°C	White, slightly shrunken	0.085	0.000
	8.2	16 month	37°C	White, slightly shrunken	0.082	0.001

Lysozyme samples were freeze-dried in pH 6.2, 7.2 or 8.2 buffer containing 3% sucrose, trehalose or dextran prior to incubation (sd, standard deviation).

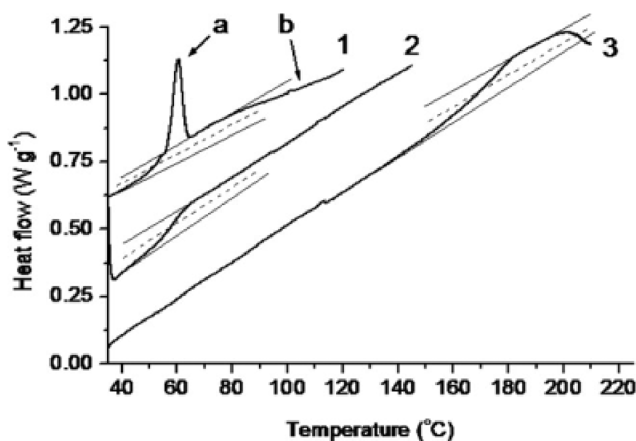

Figure 1. Differential scanning calorimeter (DSC) scans of amorphous lysozyme-carbohydrate pH 6.2 phosphate buffer mixtures to determine their glass transition temperatures (T_gs).

(1) Sucrose formulation first scan showing (A) enthalpy relaxation peak, and (B) the onset of an endothermic (buffer) crystallisation peak. (2) Trehalose formulation, rescan. (3) Dextran formulation, rescan. For further details see text.

stable with respect to buffer crystallization when heated above their glass transition temperature. For all such samples a second scan was performed thus removing the effects of physical aging on the scans which can affect the results (20). In the unique case of the sucrose pH 6.2 samples a T_g determined from the first scan as in Figure 1 is reported. All the formulations were glassy at ambient temperatures with glass transition temperatures varying in the range 37.7°C for the pH 6.2 sucrose formulation to 187.0°C for the pH 8.2 dextran formulation as shown in Table 2. The water contents were low and varied in the range 1.0–3.0% w/w (Table 2). The range of glass transition temperatures means that during incubation in the stability studies the physical state of the samples varied from being wholly in the glass state (dextran samples), largely in the glass state (trehalose samples at 90°C are in a viscous liquid state above their glass transition temperature but all other samples are in the glass state) to

Table 2. Glass transition temperature and water content of freeze-dried lysozyme-carbohydrate-phosphate buffer formulations

Carbohydrate	pH*	T_g (°C)	Water Content (% w/w)
Sucrose	6.2	37.7	1.0
	7.2	75.4	2.6
	8.2	87.3	2.5
Trehalose	6.2	61.0	1.0
	7.2	69.7	1.7
	8.2	74.7	1.2
Dextran	6.2	172.0	2.8
	7.2	182.1	2.4
	8.2	187.0	3.0

Prior to freeze-drying each sample contained 5 mg/mL lysozyme, 3% w/w carbohydrate and 0.1 M sodium phosphate buffer. pH refers to value prior to freeze-drying.

*Refers to pH upon formulation and not in the glassy state.

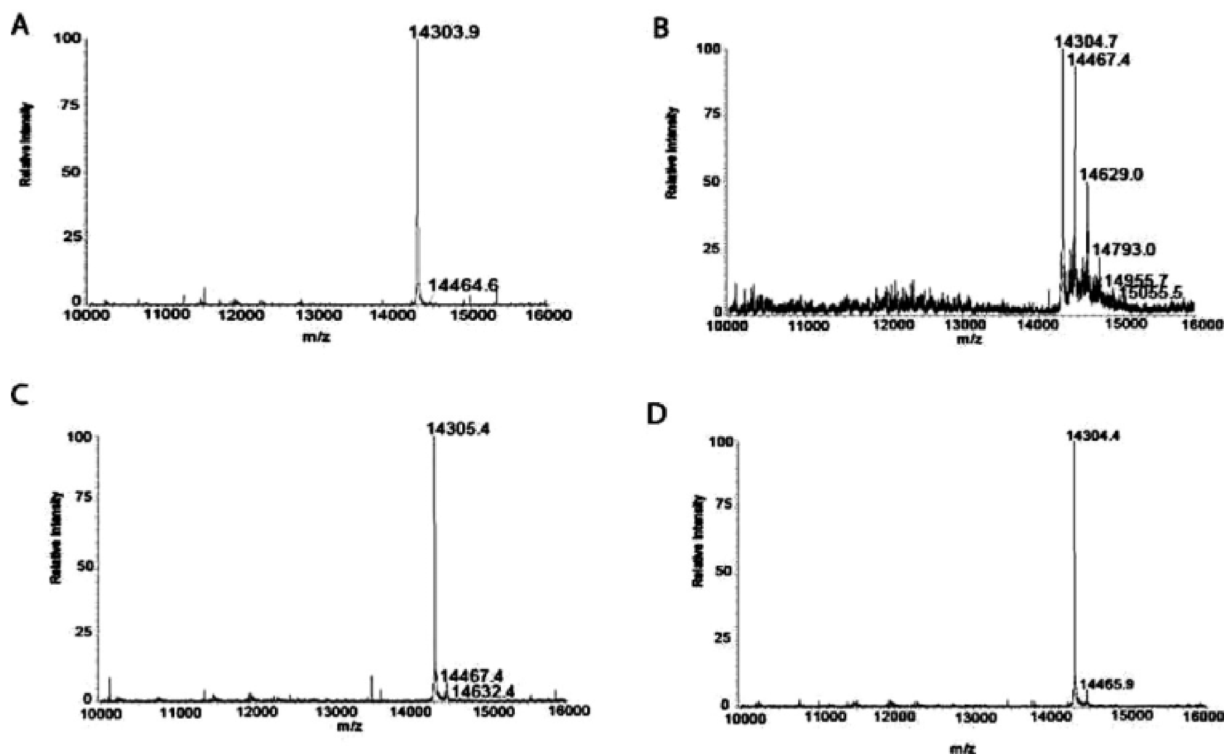

Figure 2. Electrospray ionization mass spectrometry analysis of lysozyme samples formulated in sucrose after freeze-drying and storage for 1 month at 55°C.

(A) Lysozyme formulated at pH 6.2 at time = 0 (control sample), (B) lysozyme formulated at pH 6.2 after 1 month at 55°C, (C) lysozyme formulated at pH 7.2 after 1 month at 55°C, (D) lysozyme formulated at pH 8.2 after 1 month at 55°C.

being in both viscous liquid and glass states (pH 6.2 sucrose samples at 37°C are in the glass transition region and at higher temperatures are in the viscous liquid states whereas the pH 7.2 and 8.2 samples are only in the viscous liquid states at 90°C). It should be noted that the T_gs are the initial T_gs before significant reaction/protein modification has occurred. The glycation reaction produces water as the reaction proceeds and the water content therefore increases which would be expected to depress the T_g.[23]

The effect of glass formulation variables and storage conditions upon enzymatic activity

The lytic activity of lysozyme for the bacterial substrate *Micrococcus lysodieticus* was used to determine if the enzymatic activity of lysozyme samples was preserved or degraded more efficiently/rapidly under any of the formulation and storage conditions investigated. For this analysis all activities were determined in triplicate and the average is shown in Table 1. As it can be observed from the resulting initial rates calculated and presented in Table 1, there was very little variation in the initial rate at which lysozyme cleared a solution of the bacterial substrate with changing formulation and storage conditions. The largest difference was observed when lysozyme was freeze-dried in sucrose formulated at pH 6.2 and stored at 90°C for 24 h where the initial rate was much lower than that for the other two pH's (Table 1). This trend of the lowest initial rate being observed in samples formulated at pH 6.2 when compared with those formulated at pH 7.2 or pH 8.2 was consistently observed across the majority of storage conditions (Table 1). As such, the data suggest that preformulation of lysozyme at pH 6.2 is likely to result in the greater relative loss of enzymatic activity regardless of the carbohydrate in the formulation. Further, the data show that lysozyme is a very robust enzyme that retains its activity under a wide range of conditions, even in samples that physically appear modified and therefore may not be the best choice for studying effects on enzymatic activity under such conditions.

Direct ESI mass spectrometry analysis of lysozyme samples confirms chemical modification under certain conditions

Direct ESI mass spectrometry analysis was undertaken on lysozyme samples upon freeze-drying and storage to determine the extent of any chemical modifications resulting in mass change. As shown in Figure 2A, LC-ESI-MS analysis of a lysozyme sample freeze-dried in sucrose at pH 6.2 prior to storage (time = 0) gave a single peak with a molecular mass of 14,303.9 Da in agreement with the theoretically calculated mass for intact lysozyme. Subsequent analysis of lysozyme freeze-dried in sucrose formulated at pH 6.2, 7.2, and 8.2 and stored at 55°C for 1 month showed obvious chemical modification to some of the protein material (Figures 2B–D). The deconvoluted spectrum of lysozyme samples formulated at pH 6.2 and incubated at 55°C contained four well-defined extra peaks observed at 14,467, 14,629, 14,793, and 14,955 Da, respectively (Figure 2B, Table 3) corresponding to increases in mass of multiples of 162 Da. This mass change can be assigned to protein modification resulting from the condensation of one unit of glucose or fructose with the amino group of a lysine residue on the surface of the protein. This nonenzymatic process termed protein glycation[24,25] is possible because of the hydrolysis of sucrose to yield fructose and glucose.[26] In addition to these well defined peaks, there were other less well-defined peaks/

areas within the spectrum indicative of additional chemical modifications or rearrangements that had occurred, but it was not possible to assign these from this data. It is likely that some of these products arise from rearrangement of the glycation adducts to yield advanced glycation end products (AGEs).

Mass spectrometry analysis of lysozyme samples freeze-dried in sucrose at pH 7.2 and subsequently stored at 55°C for 1 month showed less chemical modification than that observed at pH 6.2 with only two of these extra peaks present in the deconvoluted spectra; those at 14,467 and 14,632 Da, respectively (Figure 2C). The extent of chemical modification was even less in those samples formulated before freeze-drying in sucrose at pH 8.2 with just one of these major additional peaks being observed, that at 14,466 Da, suggesting that less glycation occurs under these conditions. A clear relationship between the pH before freeze-drying and protein glycation was therefore apparent. We note that the glycation of proteins can suppress or change its ionization behavior, therefore, although the relative levels of glycated and nonglycated material cannot be calculated from these data, the relative levels of each glycated product can be directly compared and thus allows the comparisons and conclusions drawn above to be made.

Table 3. LC-MS Determined Masses of Lysozyme Samples After Incubation at 55°C for 1 Month

pH	Excipient	Mass	Mass Difference	Identity/ Assignment
6.2	Sucrose	14304.7		Lysozyme
		14467.4	162.7	1 × glycation
		14629.0	324.3	2 × glycation
		14793.0	488.3	3 × glycation
		14955.7	651	4 × glycation
		15098.2	793.5	5 × glycation
7.2	Sucrose	14305.4		Lysozyme
		14467.4	162	1 × glycation
		14632.0	326.6	2 × glycation
8.2	Sucrose	14304.4		Lysozyme
		14465.9	161.5	1 × glycation
6.2	Trehalose	14304.7		Lysozyme
				1 × glycation
		14630.2	325.5	2 × glycation
7.2	Trehalose	14304.5		Lysozyme
8.2	Trehalose	14304.4		Lysozyme
6.2	Dextran	14303.6		Lysozyme
7.2	Dextran	14304.0		Lysozyme
8.2	Dextran	14304.4		Lysozyme

Lysozyme samples were freeze-dried in pH 6.2, 7.2 or 8.2 buffer containing 3% sucrose, trehalose or dextran.

When sucrose formulated samples were stored at lower temperatures but for longer periods of time a similar trend was observed (Figure 3). Sucrose samples stored at 37°C for 16 months after formulation at pH 6.2 prior to freeze-drying once again showed chemical modification of up to 4 discrete higher masses; 14,466, 14,629.5, 14,791.2, and 14,955.5 Da corresponding to glycation events (Figure 3A). In addition, various other species were once again present and detectable between the unmodified protein (14,304.7 Da) and the peaks at 14,466 and 14,629.5 Da (Figure 3A), indicative of further undefined modifications occurring. Despite these changes, the lytic activity of such samples was not changed compared with those formulated at pH 7.2 or pH 8.2 (Table 1) where there was little evidence of modification. For samples formulated in sucrose at pH 7.2 prior to freeze-drying and storage for 16 months at 37°C only one of the glycation peaks was detected (14,465.2 Da) along with a small amount of other modified material between this and the unmodified peak

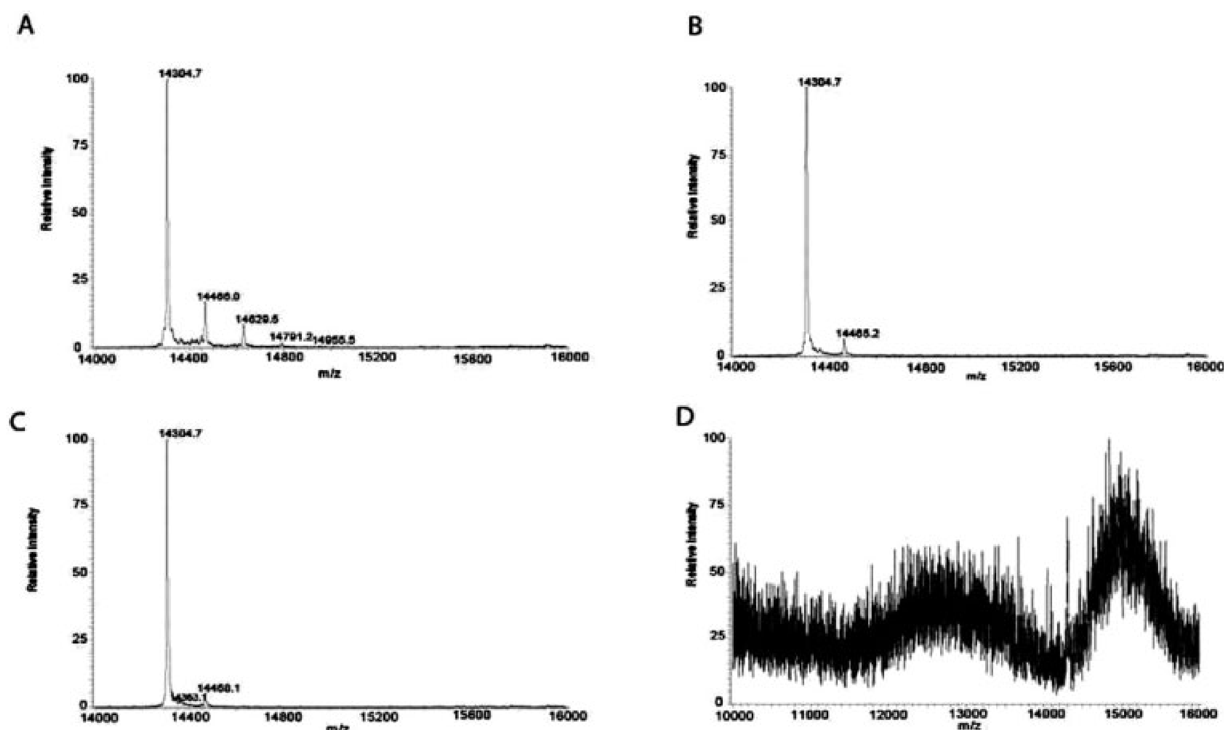

Figure 3. Electrospray ionization mass spectrometry analysis of lysozyme samples formulated in sucrose after freeze-drying and storage at various times and temperatures.

(A) lysozyme formulated at pH 6.2 after 16 months at 37°C, (B) lysozyme formulated at pH 7.2 after 16 months at 37°C, (C) lysozyme formulated at pH 8.2 after 16 months at 37°C, (D) lysozyme formulated at pH 6.2 after 24 h at 90°C.

(Figure 3B). A similar profile to that of the pH 7.2 samples was observed in those samples formulated in sucrose at pH 8.2 prior to freeze-drying and storage at 37°C for 16 months (Figure 3C).

In contrast to the previous results, when samples formulated in sucrose at pH 6.2 prior to freeze-drying were stored at 90°C for 24 h the spectra was highly variable and the main peak was observed as a broad peak at or around 15,000 Da indicative of the protein being modified to different degrees and with varying modifications (Figure 3D). It was not possible to assign any of the potential modifications from this data. Interestingly, in this sample there appears to be a further broad peak centered just below 13,000 Da suggesting degradation of some of the material in addition to those chemical modifications leading to an increase in the observed mass (Figure 3D). This agrees with the enzymatic data whereby the lytic activity of this sample was reduced compared with the samples at 90°C for 24 h formulated at pH 7.2 or 8.2 (Table 1). Together, these results confirm that the temperatures and times investigated are sufficient for accelerated stability studies in order to "force" modifications. We also note that these temperatures are used routinely within industry for such investigations.

When lysozyme was freeze-dried using trehalose as the carbohydrate and then subjected to accelerated stability studies, there was little evidence by intact mass spectrometry that chemical modification or loss of protein integrity had occurred under any of the conditions investigated (Table 3). Only those samples formulated in trehalose at pH 6.2 prior to freeze-drying and then stored subsequently at 90°C for 24 h showed readily observable, but still minor, degrees of chemical modification. This agrees with the enzymatic activity data whereby comparison of the trehalose samples stored at 90°C showed that those formulated at pH 6.2 had significantly lower enzymatic activity than those at 7.2 or 8.2 (Table 1). The deconvoluted spectra of samples freeze-dried and stored in this way showed one defined additional peak (14,628.5 Da) along with a small amount of material indicative of other chemical modification. Formulation at higher pH or more extreme storage conditions resulted in little detectable modification. For all dextran samples, there was no evidence of chemical modification under any of the conditions investigated.

Tryptic mapping confirms glycation and deamidation are the major chemical modifications to lysozyme under the freeze-dried conditions investigated

Mass spectrometry analysis of lysozyme samples following freeze-drying and storage showed conclusively that time, temperature and formulation variables determined the extent, and type, of chemical modification observed. However, although such analysis can confirm the presence of chemical modification it does not allow identification of which amino acids are modified within the protein, or the unambiguous assignment of small mass change modifications such as deamidation. Lysozyme samples that had been freeze-dried and stored at 55°C for 1 month were therefore subjected to tryptic peptide mapping in order to determine which amino acid residues were modified and to more fully characterize the observed modifications. The resulting tryptic peptides were then separated by reverse phase HPLC and ESI mass spectrometry (Figure 4 and Table 4).

The expected tryptic peptides from lysozyme were initially identified from their masses and later confirmed by tandem mass spectrometry analysis (Table 4 and Figure 4). Confirmation of glycation, and therefore the glycated lysine residue, was relatively straightforward as trypsin does not cleave after glycated lysine residues and therefore glycated peptides contain an uncleaved lysine residue.[27] In addition to an uncleaved lysine residue, glycated peptides are detected in the mass spectrometer as 162 Da greater in mass than would be expected in the absence of glycation due to the presence of the sugar residue. Modifications and their site(s) were also confirmed by tandem mass spectrometry analysis.

Glycation was observed at all six lysine (K = lysine) residues within lysozyme during this study when samples were formulated in sucrose (Table 4). Most glycation was observed at lysine residues 96 and 33 as determined from the HPLC UV traces (Figure 4). The extent of glycation varied with the formulation, with samples formulated at pH 6.2 in sucrose prior to freeze-drying giving rise to the greatest extent of modification. In samples treated in this manner 3 of the glycation sites, K^{33} in peptide T6+T7, K^{97} in T12+T13, and K^{116} in T15+T16 were visible as extra peaks in the UV traces (Figure 4). Whilst the other three glycation sites detected did not give rise to new, clearly visible peaks in the UV spectra, these were clearly present when the mass spectra data was analyzed in detail, suggesting that these sites are less susceptible to protein glycation. With increasing pH, fewer lysines were glycated and the peaks for those that were detected appeared smaller than the corresponding peaks observed in the pH 6.2 samples (Figure 4). As expected, there was much less evidence of protein glycation in trehalose formulated freeze-dried samples agreeing with the data for the intact protein (Table 4). Further, as expected there was no evidence of glycation in dextran samples in agreement with data from the intact protein mass spectrometry analysis (Table 4).

The tryptic mapping and ms/ms analysis also revealed that deamidation had occurred to lysozyme samples. Deamidation of N^{103} was observed in all samples (peaks 7h and 7j, Figure 4) and has previously been observed in control samples in other studies suggesting that this is not related to the freeze-drying or formulation variables investigated in this study.[13] However, there was an additional deamidation peak corresponding to N^{103} which was only detected in samples formulated in sucrose at pH 6.2 prior to freeze-drying (Table 4). There was no evidence of other commonly observed modifications such as methionine oxidation, however, there were peptide peaks and masses within these spectra which we were not able to assign on the basis of mass alone and the appearance of several of these did exhibit formulation dependent tendencies suggesting that additional, but as yet uncharacterized modifications, do arise under the variables investigated.

Discussion

The integrity of protein based products such as biotherapeutic pharmaceuticals (e.g. monoclonal antibodies) must be preserved prior to administration in order to prevent immunogenicity and maintain bioactivity. Similarly, the structure of proteins in food ingredients must be maintained in order to retain their functionality prior to use. Plant anhydrobiotes can preserve biological activity of proteins by accumulating sugars in their tissues during drying and transforming the cytoplasm into a glassy state.[28] A similar approach has been applied to the stabilization of high-value therapeutic proteins

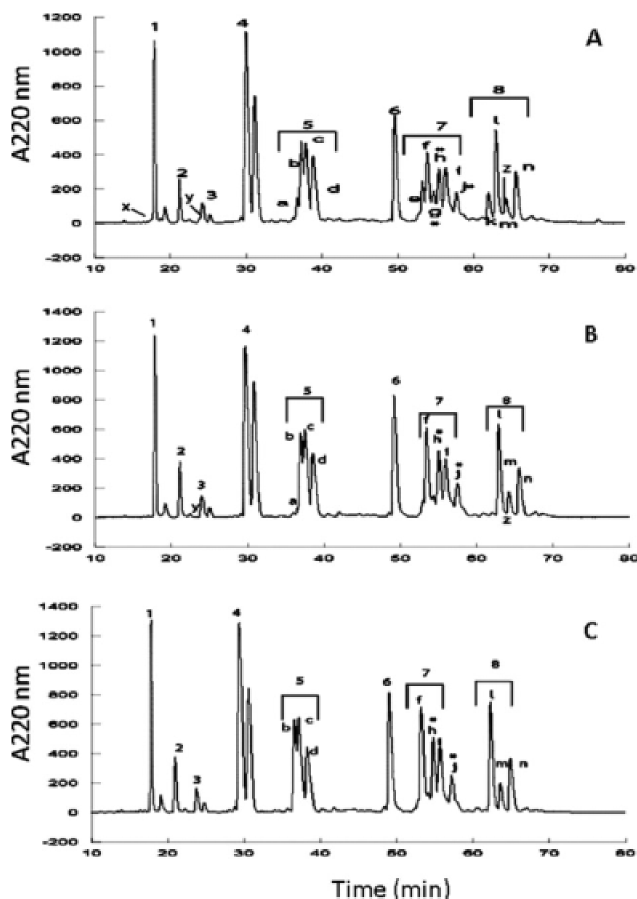

Figure 4. Reversed-phase HPLC separation of the tryptic peptides of lysozyme formulated (A) sucrose at pH 6.2 before freeze-drying and storage at 55°C for 1 month, (B) sucrose at pH 8.2 before freeze-drying and storage at 55°C for 1 month, and (C) trehalose at pH 6.2 before freeze-drying and storage at 55°C for 1 month.

The peptides are labeled to correspond to the peaks and assignments listed in Table 4. Deamidation modifications are depicted with a star (*).

whereby they are preserved in the glassy state following freeze-drying in a carbohydrate based formulation.[18] There has been considerable work undertaken on how formulation and storage temperature of protein glasses affects the rates at which proteins aggregate or lose their biological activity; however, our understanding of the molecular mechanisms of protein stabilization and chemical modification in glasses is incomplete. We, therefore, investigated the relationship between chemical modification and rate thereof with variation of pH, temperature, and the glass transition temperature using the most commonly used carbohydrate additive for the formation of mixed protein–carbohydrate glasses (sucrose) as well as trehalose and dextran to further our understanding of the mechanisms and processes involved.

The glass transition temperature of these mixed formulations depends upon all the compositional variables i.e., the amount of carbohydrate, monosodium phosphate, disodium hydrogen phosphate, lysozyme, and water. The relative amount of the phosphate salts depends upon the buffer's pH. Whilst the present dataset is insufficiently large to distinguish effects of all the variables there was a clear difference between the T_g of the disaccharide-based formulations (sucrose, trehalose) and the higher molecular weight polysaccharide-based formulations (dextran) which reflects the higher glass transition temperature of the polysaccharide excipient in its pure dry amorphous form which has been reported as 189°C for dextran[29] when compared with 67 and 111°C for sucrose[30] and trehalose[31] respectively. The sodium phosphate buffer, water content and the protein itself have secondary effects on the T_g of the freeze-dried formulations. The T_g's of all the formulations increase with pH in agreement with data reported by Ohtake *et al*.[32] who reported that sucrose- and trehalose-phosphate buffer formulations dried from higher pH (richer in disodium hydrogen phosphate) have higher T_g's. We note that the relatively low T_g of the sucrose pH 6.2 formulation has a composition which is in the vicinity of a phase separation[32] and that in samples formulated at pH 7.2 and 8.2 the T_g's of sucrose were higher than those of trehalose, reversing the relationship between these observed at pH 6.2. The pH 7.2 and 8.2 sucrose formulations have T_g's above that of pure dry sucrose indicating a positive contribution of the buffer and protein to the overall glass transition temperature of the mixture despite the presence of water which generally acts as a plasticiser, depressing the glass transition temperature of the mixture.[33]

The relationship between T_g, carbohydrate, pH of formulation, temperature of storage, physical appearance, and the degree of chemical modification observed is summarized in Figure 5. The observed physical structure of the freeze-dried formulations after incubation at elevated temperatures (Table 1) were directly related to the changing material properties of these amorphous materials as they were heated through their glass transitions (Table 2 and Figure 5A). At temperatures in excess of T_g, the materials transform from a solid-like glass to a highly viscous liquid. The viscosity of the liquid decreases rapidly with increasing temperature such that within 10°C of T_g the material is sufficiently soft for surface tension to cause the initially porous low density structure to rapidly collapse, losing much of its porosity and forming a compact high density liquid structure.[34] This collapse is a kinetic process, its rate predicted to be proportional to the viscosity of the liquid phase.[35] With prolonged storage, as in the 16 month incubation at 37°C, the collapse effect is observed at temperatures in the region of the glass transition itself as is observed for the sucrose pH 6.2 formulation (Table 1).

There have been a number of studies that have investigated the effect of different buffer formulations on protein integrity, however, in most cases overall changes in secondary structure have been studied using techniques such as FTIR but detailed studies of any chemical modifications are rare. Also, the linking of formulation to activity is not common.[5,11,36] There was an obvious change in lytic activity only in those lysozyme samples formulated in pH 6.2 buffer and heated for 24 h at 90°C (Table 2). However, here we have shown that while changes in activity are not always measurable (Table 2), there may be chemical modification to the protein (Table 3) occurring that could potentially lead to changes in the antigenicity of the protein.

We note that both chemical and physical variables affect the rate and extent of protein modifications in accelerated thermal stability tests of freeze-dried formulations. While the effects of some of the chemical and physical variables can be distinguished, others could not be due to the variables being correlated. The chemical variable is the nature of the excipient (sucrose, trehalose, dextran) and the physical variables are temperature, pH (prior to freeze-drying) and the physical state of the formulation (nonglassy/glassy, viscosity, etc.). While the effect of the change in pH on the rate of

Table 4. Identification and Assignment of Peaks Observed in the LC-MS Spectra Following Tryptic Digestion of Lysozyme Samples After Incubation at 55°C for 1 Month

Peak No.*	Calculated Mass	Observed Mass	ms/ms†	Peptide‡	Modification	Site	Sucrose (pH 6.2)	pH 7.2	pH 8.2	Trehalose (pH 6.2)	pH 7.2	pH 8.2	Dextran (pH 6.2)	pH 7.2	pH 8.2
X	768.37	768.4	N	T1+T2	Glycation	K1?	X								
1	606.4	606.5	N	T1+T2			X	X	X	X	X	X	X	X	X
	874.4	874.5	Y	T5			X	X	X	X	X	X	X	X	X
2	1428.65	1428.8	Y	T7			X	X	X	X	X	X	X	X	X
Y	1212.5	1212.6	N	T3+T4	Glycation	K13?	X	X	X						
3	1050.5	1050.7	Y	T3+T4			X	X	X	X	X	X	X	X	X
	894.4	894.5	Y	T3			X	X	X	X	X	X	X	X	X
4	1492.6	1492.7	Y	T9+T10			X	X	X	X	X	X	X	X	X
	994.4	994.6	Y	T9			X	X	X	X	X	X	X	X	X
5a	1496.7	1496	Y	T15+T16	Glycation	K116	X	X	X						
5b	1334.7	1334.7	Y	T15+T16			X	X	X	X	X	X	X	X	X
5c	1045.5	1045.7	Y	T16			X	X	X	X	X	X	X	X	X
5d	1753.83	1753.8	Y	T8			X	X	X	X	X	X	X	X	X
6	1326.6	1326.7	Y	T6			X	X	X	X	X	X	X	X	X
7e	1966.9	1966	Y	T12+T13	Glycation	K 97	X								
7f	1803.9	1803.8	Y	T12+T13			X	X	X	X	X	X	X	X	X
7g	1803.9	1804.6	Y	T12+T13	Deamidation	N103	X								
7h	1803.9	1804.6	Y	T12+T13	Deamidation	N103	X	X	X	X	X	X	X	X	X
7i	1675.8	1676.8	Y	T13			X	X	X	X	X	X	X	X	X
7j	1675.8	1677.5	Y	T13+	Deamidation	N103	X	X	X	X	X	X	X	X	X
8k	2898.2	2898	Y	T6+T7	Glycation	K 33	X	X	X	X	X				
8l	2736.2	2736.6	Y	T6+T7			X	X	X	X	X	X	X	X	X
z	2801.2	2804.4	Y	T11+T12	Glycation	K96	X	X	X						
8m	2639.2	2639.4	Y	T11+T12			X	X	X	X	X	X	X	X	X
8n	2511.1	2511.4	Y	T11			X	X	X	X	X	X	X	X	X

The lysozyme samples were freeze-dried in pH 6.2, 7.2 or 8.2 buffer containing 3% sucrose, trehalose or dextran prior to incubation.

*Refers to peaks marked in Figures 5 and 6. † Y = yes, N = no for confirmation of peptide and modification by ms/ms sequencing. ‡ Refers to tryptic peptides generated from N-terminal end of lysozyme with first peptide being T1.

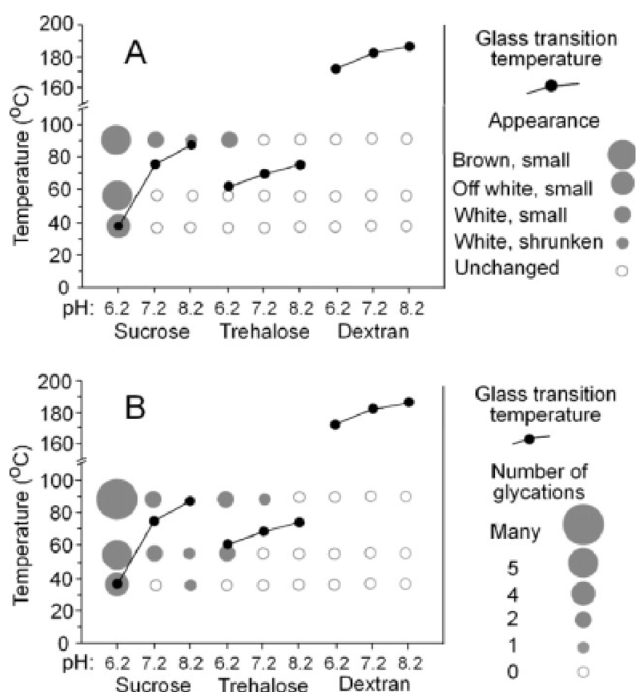

Figure 5. The relationship between the carbohydrate excipient (sucrose, trehalose or dextran), storage condition (37°C for 16 months, 55°C for 1 month, 90°C for 24 h), glass transition (T_g) and (A) physical appearance of samples, and (B) degree of glycation observed.

The physical appearance and degree of glycation are most obviously affected in samples above the T_g, however modification and changes to the physical appearance were observed in both sucrose and trehalose samples below the T_g indicative of changes occurring in the glass. For further details see text.

protein modification may be causal, another factor to be considered is the physical state of the formulations. The 3% sucrose pH 6.2 formulation has a glass transition temperature of 37.7°C and so this system is in the vicinity of the glass transition at 37°C and in a viscous liquid state at 55 and 90°C and this is the system which reacts most extensively. Several of the glassy systems (sucrose at pH 7.2 and 8.2 and trehalose at pH 6.2 and 7.2) show more limited reaction (Tables 3, 4 and Figure 5B). For the sucrose formulations the pH variable is correlated with the changing physical state, for example at 55°C as the pH increases from 6.2 to 8.2 the systems state changes from a viscous liquid state (pH 6.2, T_g 37.7°C) to a glass (pH 7.2, T_g 75.4°C and pH 8.2, T_g 87.3°C). However, although the chief chemical modification observed (glycation) was more abundant in the sucrose sample formulated at pH 6.2 at temperatures when it would no longer be expected to be glassy, modification was clearly occurring in sucrose and trehalose samples that were in the glassy state (Figure 5B).

Although deamidation was detected across most of the formulations and variables investigated (Table 4), the most prominent modification was protein glycation. Glycation results from reaction with glucose and/or fructose which in the formulations investigated here is the product of degradation of the carbohydrate excipients. The degradation process generating the reducing sugars depends upon the nature of the excipient, temperature and pH such that the levels of reducing sugar generated followed the trend sucrose > trehalose > dextran. We note that pH directly affects the rate of glycation as it changes both the rate of sucrose hydrolysis and the nucleophilicity of lysine residues. On this basis of the extent of glycation in our formulations follows from the concentration of reducing sugars present. In addition to the identity of the carbohydrate excipient the protein modifications vary with the pH of the formulations. For both the sucrose and the trehalose formulations the extent of protein modification, as shown from the results of the LC-MS of the intact lysozyme (e.g. Table 3) and the peptide mapping (e.g. Table 4), increase as the pH decreases from 8.2 through 7.2 to 6.2. At 55°C, the trend occurs for both the trehalose formulations which are all in a solid glassy state and for the sucrose formulations which are glassy at pH 8.2 and 7.2 and are in a viscous liquid state at pH 6.2.

The peptide mapping results shown in Table 4 reveal that the glycation of lysine residues varies with site although all six residues are glycated under at least one of the conditions investigated. Although it is difficult to precisely determine the different lysine reactivities from this data, lysines 1 and 97 appear to be the least reactive under the conditions investigated whilst lysine 33 appears to be the most such that the reactivity varies in the order $K^{33} > K^{13}, K^{116}, K^{96} > K^{1}, K^{97}$. Although reactions of K^1 and K^{97} occurred solely in the liquid pH 6.2 sucrose formulations, the other residues showed reaction in the glass state. Lysine residue K^{33} is presumably the most reactive with glycation occurring at this site preferentially in the trehalose formulations. This order differs from that previously reported for lysine glycation of lysozyme in solution whereby K^{97} was the most reactive. Further, K^{97} is part of a di-lysine motif and it is generally accepted that these are more susceptible to glycation due to local acid–base catalysis making one residue more nucelophilic.[36] The fact that K^{97} was least reactive in the freeze-dried samples investigated here suggests that the local structural environment, accessibility, and charge of the lysine residues is different to that found in solution and crystal forms. Further studies to elucidate such differences between the solution and glassy state are therefore required and we note that previous studies in solution using peptide models have shown that the local environment does influence the rate of glycation and local secondary structure elements on glycation.[37]

Conclusions

Considering all the data together, we have described the characterization of two aspects of stability, chemical stability characterized using mass spectrometry, and the maintenance of biological activity characterized using an enzyme activity assay. We have studied these, both above and below the glass transition of initially amorphous formulations. Perhaps most importantly, we have identified that the rate of protein modification and preferential sites for reaction differ between the solution and glassy state, at least for nonenzymatic glycation. This has implications for predicting likely protein modifications, and sites thereof, to proteins in the glassy state from data previously gathered in the solution state. Further, although dextran formulated samples appeared to be the most stable by our criteria, practically these samples were difficult to resolubilized/reconstitute, which often took several hours rendering them impractical commercially. There were also clear trends between protein modification and buffer pH prior to freeze-drying and the primary modification detected, glycation, however the differences in the apparent reactivities of the lysine residues for glycation after freeze-drying compared with those previously determined in solution has clear implications for the prediction of potential

modifications in the glassy state. Further, we predict that whilst the relationship between formulation variables, T_g and the physical attributes of proteins in the glassy state is likely to hold for the majority of proteins, that the relationship between formulation variables and chemical modification will change as a result of the different environments that individual amino acids experience on freeze-drying.

Acknowledgments

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