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Stability of crystallised and spray-dried lysozyme

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Abstract

Moisture and temperature promote protein degradation. The stabilities of commercial, crystallised and spray-dried lysozyme, a model protein, were assessed under these stresses to explore whether a crystalline protein had better storage stability than a conventionally produced one. Samples were maintained at different relative humidities (RH) and temperatures for 20 weeks and stabilities estimated in solid and aqueous states. Differential scanning calorimetry (DSC) and thermogravimetry (TGA) characterised solid samples. Fourier transform Raman (FT-Raman) spectroscopy analysed solid material and aqueous solutions. High sensitivity differential scanning calorimetry (HSDSC) and enzymatic assays were used to monitor solutions. DSC and HSDSC data revealed that crystals maintained thermal stability at high RH; spray drying appreciably changed melting characteristics. These results correlated with enzymatic assays that demonstrated good activity retention for crystals but less so for spray-dried material (e.g. 95 and 87% relative to fresh samples after 20 weeks at 40 °C/75% RH). FT-Raman analysis showed that crystallised lysozyme better-maintained protein conformational integrity compared to spray-dried samples in accelerated stability studies. Based on TGA data, spray-dried protein absorbed water on storage under humid conditions, which induced instability. Thus, crystallisation enhanced storage stability of lysozyme with negligible loss of activity.

Keywords: Lysozyme; Crystallisation; Spray-dried; Stability; Calorimetry; Raman

1. Introduction

Stabilisation of proteins during storage is important as maintaining their native structure represents a critical challenge in protein formulation. Degradation pathways of proteins, physical or chemical, should be minimised during processing to produce protein formulations with suitable integrity and shelf life (Tracy, 1998; Wang, 1999, 2000). Previously, Elkordy et al. (2002) discussed the effects of processing techniques (crystallisation and spray drying) on conformational integrity and activity of lysozyme, a model protein. Lysozyme crystals were more stable and active than a spray-dried form when initially analysed thermally and spectroscopically.

Many factors affect the stability of proteins during storage; critical are moisture and temperature. Instabilities of protein pharmaceuticals, like other pharmaceutical products, are detected after prolonged storage under normal conditions. Accordingly, the exposure of protein formulations to elevated temperatures and/or humidities can accelerate the instability of the product and this technique helps assessment of the protein shelf life. Many reports cited the impact of temperature (e.g. Volkin and Middaugh, 1992) and moisture (Chang et al., 1993; Bell et al., 1995; Maa et al., 1998; Andya et al., 1999) on spray-dried or lyophilised solid state protein (containing stabilis-

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ing excipients to protect protein from denaturation at the gas–liquid interface and thermal stress produced from drying methods). The literature concluded that the chemical stability of protein decreases with increasing moisture content in the solid as a result of conformational changes and increasing water mobility within the powder. These processes degrade protein and reduce activity. It is worth noting that although the dried solid state protein products contained stabilising additives, they were affected by elevated relative humidity. Maa et al. (1998) proposed that protein powder should be manufactured and processed under the driest environment that manufacturing facilities allow (usually less than 20% RH).

Water is important in maintaining conformational stability and biological activity of proteins (Shah and Ludescher, 1993; Allison et al., 1999). Thus, a water layer surrounding proteins maintains folded conformations through van der Waals' interactions, salt bridges and hydrogen bonds. The maximum hydration level to stabilise proteins was 0.25 g H_2O/g protein (Shah and Ludescher, 1993; Sartor et al., 1994); above this concentration water acts as a plasticizer and molecular mobility increases. Hence, the protein dynamically changes from a glassy, solid state to the rubbery and then liquid form (Ahlneck and Zografi, 1990; Buitink et al., 2000). Consequently, the effect of hydration on the stabilisation of protein decreases with an increase in the degree of hydration (Zaks, 1992).

Shenoy et al. (2001) recently prepared crystalline and lyophilised glucose oxidase and lipase with and without the excipients trehalose, sucrose and lactitol, and they measured biological activity, protein conformations (using FT-Raman) and aggregation (via size-exclusion chromatography) in accelerated stability studies at 50 and 40 °C/75% RH. They demonstrated that dry crystalline formulations of proteins are more enduring than their amorphous forms. Here, we study another protein, crystallised without excipients. Additionally, the amorphous form we employ for comparison was obtained using spray drying (not lyophilisation). Furthermore, in this study, our formulations are exposed to four different conditions of relative humidity and temperature. A recent review article (Jen and Merkle, 2001) proposed that protein crystals may represent a valuable formulation approach for the stabilisation and delivery of protein therapeutics. Also, the publication showed that protein crystals can provide better physical handling stability and sustained release. However, Pikal and Rigsbee (1997) reported an exception to the above. They investigated insulin instability in crystalline and amorphous solids stored at different temperatures and relative humidities. They concluded that freeze-dried insulin was more inert than crystalline protein under all conditions investigated.

Our current work studies the influences of temperature and moisture on the responses of crystallised and spray-dried lysozyme after exposure to these stresses for 20 weeks to follow-up formulations after processing and to indicate whether protein crystals or a spray-dried forms have the desired stability. We employ thermal analysis (differential scanning calorimetry, thermogravimetry and high sensitivity differential scanning calorimetry) to investigate thermodynamic behaviour; FT-Raman spectroscopy to detect conformational changes; and biological assay to study catalytic activity. During storage, samples of unprocessed and processed lysozyme were analysed for secondary structure using FT-Raman.

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme in the form of lyophilised powder (purity 95, 5% sodium chloride and sodium acetate), sodium chloride (99.5%), sodium phosphate (99.3%) and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Company (St. Louis, MO). Sodium acetate anhydrous (98%) and potassium dihydrogen orthophosphate (>99%) were obtained from BDH Chemicals Ltd., Poole, UK. Water was deionised and double distilled.

2.2. Preparation of crystallised lysozyme

Lysozyme was crystallised using a published method (Elkordy et al., 2002). Crystals formed were filtered, dried and kept in a freezer (−15 °C) until the stability study began.

2.3. Preparation of spray-dried lysozyme

Lysozyme solution (1 g/100 ml distilled water) was spray-dried according to the procedure and conditions

detailed in Elkordy et al. (2002). The dried powder was secured in vials over silica gel in a freezer (−15 °C) until use.

2.4. Storage conditions

Unprocessed, spray-dried and crystallised samples of lysozyme were maintained for 20 weeks at: 4 °C/2% RH; 20 °C/65% RH; 30 °C/65% RH; 40 °C/75% RH and 60 °C/7% RH.

Samples were analysed at 0 and 20 weeks, i.e. pre- and post-storage. Also, solid samples were analysed every 4 weeks for enzymatic activity and secondary structure (using FT-Raman).

2.5. Enzymatic assay

The activity of lysozyme was assayed by monitoring the hydrolysis of a bacterial suspension (20 mg%) of *M. lysodeikticus* in phosphate buffer 0.067 M, pH 6.6, at 25 °C. Enzyme solutions (15 μg/ml) of unprocessed, spray-dried and crystallised lysozyme were prepared in the same buffer. Addition of 0.5 ml of each enzyme solution to 2.5 ml of the bacterial suspension initiated the biological reaction (Gorin et al., 1971). The decrease in the absorption rate at 450 nm was monitored using a UV-Vis spectrophotometer (Pu 8700, Philips, UK). A unit of activity decreases the absorbance at 450 nm at 0.001 min^{-1} under the above conditions. The activity was determined from: activity (units/mg) = $\Delta 450_{nm/min}/0.001$ mg enzyme in reaction mixture (Shugar, 1952). The activities of all samples were measured relative to that of a corresponding fresh sample. For example, the biological activity of the spray-dried form was measured relative to that of a fresh spray-dried material (before storage).

2.6. Powder X-ray diffraction

X-ray diffraction by crystalline substances provides information on all possible atomic spacing or crystal lattices (Brittain et al., 1991). Diffraction from a powder surface forms a pattern consisting of peaks detected at various scattering angles. With respect to proteins, a small angle X-ray diffractometer is used to determine the three dimensional structure of proteins, but in this work a large angle diffractometer was employed to detect the differences in diffraction patterns between unprocessed, spray-dried and crystallised lysozyme. A Siemens D500 X-ray powder diffractometer (Siemens, Germany) was used to generate X-ray diffractograms of samples. A copper X-ray source provided divergent beam monochromator Kα radiation of wavelength 0.154 nm. All data were collected between 2θ of 3 and 72.

2.7. Thermogravimetric analysis (TGA)

TGA determined the moisture contents of lysozyme samples pre- and post-storage. Analysis employed a TGA 7 (Perkin-Elmer Ltd., Beaconfield, Bucks). Solid samples (3–7 mg) were loaded on an open platinum TGA pan suspended from a microbalance and heated from 25 to 275 °C at 10 °C/min. To avoid moisture from air, the sample chamber was purged with dry nitrogen.

2.8. Differential scanning calorimetry (DSC)

A Perkin-Elmer differential scanning calorimeter 7 (DSC 7, Perkin-Elmer Ltd.) analysed materials pre- and post-storage. Solid samples (3–7 mg), sealed in aluminium DSC pans with vented lids and loaded in sample cells under nitrogen, were scanned from 25 to 275 °C at 10 °C/min. The instrument was calibrated using a pure indium standard.

2.9. FT-Raman spectroscopy

The FT-Raman spectra of unprocessed and processed samples were collected for solid samples (every 4 weeks and pre- and post-storage) and aqueous solutions (pre- and post-storage) using a Bruker IFS 66 (Karlsruhe, Germany) spectrometer with FRA 106 FT-Raman module equipped with a Nd^{3+}: YAG laser emitting at 1064 nm. Solids were analysed in stainless steel cups; 8% w/v solutions (high concentration used to detect and reveal well resolved Raman bands) were assessed in a quartz cuvette with a mirror surface. The laser power was approximately 200 and 328 mW for solid unprocessed and processed samples, respectively, and 900 mW for solutions. Spectra were averages of 400 scans for solids and 5000 for solutions at 4 cm^{-1} resolution over the range 3500–500 cm^{-1}, at 25 ± 1 °C.

2.10. High sensitivity differential scanning calorimetry (HSDSC)

Solution samples (pre- and post-storage) were analysed with a Microcal MCS differential scanning calorimeter (Microcal Inc., MA, USA). The calorimeter was calibrated for heat capacity using electrical pulses of known power and for temperature using sealed hydrocarbon standards of known melting points. Degassed samples (5 mg product/1 ml 0.1 M sodium acetate buffer, pH 4.6) and reference (0.1 M sodium acetate buffer, pH 4.6) were loaded into cells using a gas tight Hamilton 2.5 ml glass syringe. The sample and reference were heated from 20 to 90 °C at 1 °C/min under 2 bar nitrogen pressure. A base line, run before each measurement by loading the reference in both the sample and reference cells, was subtracted from the protein thermal data. The excess heat capacity was normalised for lysozyme concentration. Data analysis and deconvolution used ORIGIN DSC data analysis software.

All experiments were run in triplicate and the Student's *t* test was used as a test for significance.

3. Results and discussion

3.1. Enzymatic assay

Table 1 details the percentage enzymatic activity of reconstituted lysozyme samples (relative to the aqueous solution of fresh corresponding protein formulation) post-storage for 20 weeks. From Table 1, it is apparent that spray-dried lysozyme was the least stable form and that the crystallised product possessed the greatest stability. After storage for 20 weeks at 30 °C/65% RH, unprocessed lysozyme retained its biological activity. However, the recovered activity at elevated RH (75%) and temperature (60 °C) significantly decreased ($P < 0.05$). The activity of spray-dried lysozyme decreased ($P < 0.05$) at high RHs such as at 65 and 75%. The activity results of spray-dried samples kept at elevated temperature (60 °C) indicated that this form is substantially affected ($P < 0.001$) by temperature.

The results of spray-dried and crystallised lysozyme after storage at 40 °C/75% RH (Table 1) suggested that the activity of spray-dried material was significantly less than that of a crystallised form. This is because spray-dried powder took up more water (see TGA results) than was adsorbed on the surface of protein crystals (as an amorphous solid has greater free volume and molecular disorder, Hancock and Zografi, 1997). This probably led to changes in the mobility of protein molecules and subsequent decrease in activity (Separovic et al., 1998). X-ray diffractograms of unprocessed and processed samples confirmed that spray-dried lysozyme was more amorphous than crystallised protein (data not shown). Fig. 1 displays the activity of unprocessed and processed lysozyme retained after storage at 40 °C/75% RH at various time points over a 20-week period. The figure indicates that lysozyme crystals are most stable at this most challenging condition following 20-week maintenance. Unprocessed material started to lose a significant amount of activity (~8%) after 16 weeks, whilst the spray-dried formulation lost ~8% of its activity after only 12 weeks.

Table 1 illustrates the stability of the lysozyme structure within crystals even at elevated RH and

Table 1
Enzymatic activity of lysozyme samples post-storage (20 weeks)

Process	% activity				
	4 °C	20 °C	30 °C	40 °C	60 °C
	2% RH	65% RH	65% RH	75% RH	7% RH
Unprocessed	99 (1.5)	99 (2.1)	100 (2.1)	89* (4.0)	91* (1.5)
Crystallised	101 (3.1)	101 (3.6)	98 (2.6)	95 (2.6)	94* (2.1)
Spray-dried	103 (1.0)	93 (3.0)	94* (2.6)	87* (3.2)	79** (2.3)

Data are expressed as percentage of corresponding fresh samples. Values within parenthesis are S.D., $n = 3$.
* $P < 0.05$ compared to fresh sample.
** $P < 0.001$ compared to fresh sample.

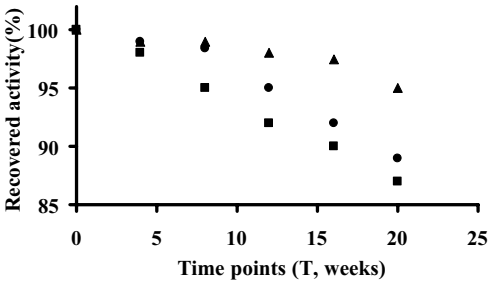

Fig. 1. Recovered activity of (●) unprocessed, (■) spray-dried and (▲) crystallised lysozyme samples after: $T = 0$, 4, 8, 12, 16 and 20 weeks storage at 40 °C/75% RH. Activity measured relative to corresponding fresh sample, $n = 3$.

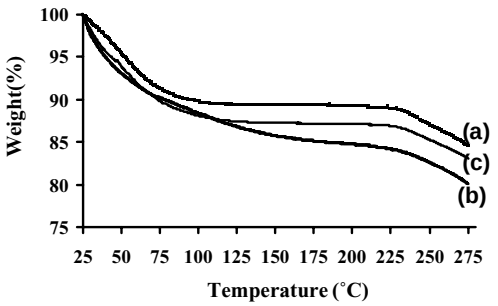

Fig. 2. Example TGA thermograms of (a) unprocessed, (b) spray-dried and (c) crystallised lysozyme stored at 40 °C/75% RH for 20 weeks. Heated from 25 to 275 °C, at 10 °C/min.

temperature following 20 weeks storage. This may be explained by the packing of protein molecules within crystals. Water molecules occupy well determined sites inside and at the surface of biological macromolecules and form a hydration shell that is important in maintaining the structural stability of active site clefts of proteins and hence in enzyme–substrate interactions (Shah and Ludescher, 1993). In our study, the lysozyme crystals contain water in their lattice that is essential to maintain the proper size of the active site cleft; consequently the enzyme-binding region fits the substrate upon reconstitution. Moreover, Frey (1994) found that lysozyme crystals prepared in a low humidity environment reduced the cell volume of crystals and changed the substrate-binding region. Consequently, activity was lost, as water is important in determining the forces between the two domains that define the active site cleft of the enzyme (Rupley et al., 1983). This explanation is consistent with the results of spray-dried lysozyme aged at 60 °C/7% RH (Table 1), as dehydration removed essential water molecules from the active site contributing to loss of biological activity even after reconstitution.

3.2. Thermogravimetric analysis (TGA)

Moisture content alters protein stability in the solid state. Accordingly, the water contents of unprocessed and processed lysozyme after storage at different humidities and temperatures were determined via TGA. Fig. 2 shows examples of TGA thermograms of materials kept at 40 °C/75% RH for 20 weeks and Table 2 summarises the data. Elkordy et al. (2002) proposed that TGA thermograms of fresh unprocessed, crystallised and spray-dried lysozyme were characterised by three stages of weight loss. The first was attributed to removal of adsorbed surface water; the weight decreased slightly in the second stage (most water removed); while in the third step weight fell because of decomposition occurring around the melting temperature. Hence, the TGA scan shows the modes of water interaction with the protein.

Table 2 shows that increasing the relative humidity from 2 to 75% RH raised the water content, especially apparent from the first stage results, for all samples. Spray-dried material absorbed more moisture compared to unprocessed and crystallised samples at all conditions. This arises from the fact that spray-dried materials are hygroscopic and can pick up moisture even at low RH. Moisture entraps, penetrates into the solid and plasticises it (Ahlneck and Zografi, 1990). At 20 °C/65% RH, all samples showed higher water contents compared with 30 °C/65% RH. Thus, moisture uptake by samples was less at 30 °C/65% RH than at a lower temperature.

With respect to temperature, Table 2 reveals that unprocessed and crystallised lysozyme stored at 60 °C/7% RH showed no significant increase in weight loss of all three stages compared to their fresh samples. Notably, the spray-dried form showed a significantly increased ($P < 0.001$) weight loss (compared to its fresh sample), especially during the third period (decomposition): this finding supports its loss of activity (cf. Table 1). Therefore, the high weight loss associated with decomposition from TGA data showed that the spray-dried lysozyme was

Table 2
Weight loss of lysozyme, stored for 20 weeks, determined by thermogravimetric analysis upon heating from 25 to 275 °C at 10 °C/min

Process	Stages of weight loss	Weight loss (% w/w)					
		Fresh	4 °C	20 °C	30 °C	40 °C	60 °C
			2% RH	65% RH	65% RH	75% RH	7% RH
Unprocessed	1	4.65 (0.51)	3.35 (0.21)	8.25 (0.39)	7.08 (0.21)	9.73 (0.41)	2.78 (0.40)
	2	1.62 (0.39)	0.92 (0.12)	1.26 (0.11)	1.39 (0.19)	1.05 (0.10)	1.59 (0.51)
	3	5.20 (0.31)	5.33 (0.29)	4.97 (0.39)	4.31 (0.21)	4.26 (0.39)	5.70 (0.31)
	Total	11.47	9.6	14.48	12.78	15.04	10.07
Crystallised	1	5.80 (0.56)	1.89 (0.39)	8.89 (0.38)	8.20 (0.19)	10.9 (0.31)	1.23 (0.12)
	2	2.10 (0.66)	1.71 (0.19)	1.64 (0.21)	1.60 (0.41)	1.46 (0.20)	2.55 (0.30)
	3	4.41 (0.37)	4.40 (0.30)	4.40 (0.21)	4.15 (0.11)	4.59 (0.39)	5.61 (0.35)
	Total	12.31	8.00	14.93	13.95	16.95	9.39
Spray-dried	1	4.20 (0.21)	7.38 (0.31)	9.86 (0.31)	9.51 (0.58)	11.56 (0.21)	3.41 (0.25)
	2	2.19 (0.11)	1.60 (0.28)	1.57 (0.21)	2.56 (0.11)	0.87 (0.21)	2.83 (0.41)
	3	4.16 (0.22)	4.55 (0.66)	6.21 (0.57)	3.23 (0.20)	6.22 (0.18)	17.22** (0.61)
	Total	10.55	13.53	17.64	15.30	18.65	23.46

Values within parenthesis are S.D., $n = 3$.
** $P < 0.001$ compared to fresh sample.

detrimentally affected by temperature compared with unprocessed and crystallised protein.

3.3. Differential scanning calorimetry (DSC)

Typical DSC traces of unprocessed, crystallised and spray-dried lysozyme after storage for 20 weeks at 4 °C/2% RH and 40 °C/75% RH are illustrated in Fig. 3A and B, with numerical data in Table 3. The broad endotherm at ~55–100 °C was larger and shifted to a lower temperature with increasing RH, indicative of the increase in water content of the samples.

Interestingly, Table 3 shows a significant increase (for unprocessed and crystallised protein kept at

Table 3
Apparent denaturation temperature (T_m) of lysozyme pre- and post-storage (20 weeks); differential scanning calorimetry (DSC)

Conditions		T_m (°C)		
°C	% RH	Unprocessed	Crystallised	Spray-dried
Fresh		200.3 (0.40)	195.3 (0.63)	205.9 (0.60)
4	2	200.5 (0.72)	195.1 (0.56)	204.2 (0.35)
20	65	203.8* (0.81)	200.8* (0.90)	206.3 (0.30)
30	65	204.5** (0.45)	201.0* (0.30)	205.9 (0.21)
40	75	205.6** (0.71)	198.5* (1.12)	207.4* (0.47)
60	7	Split endotherm	192.7 (0.40)	Not detected

Values within parenthesis are S.D., $n = 3$.
* $P < 0.05$ compared to fresh sample.
** $P < 0.001$ compared to fresh sample.

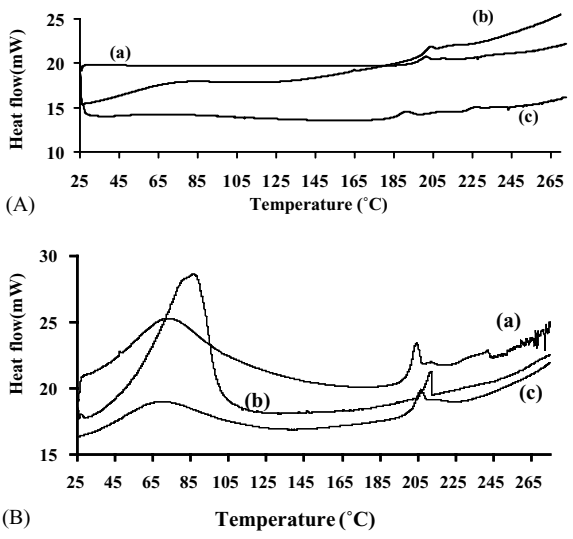

Fig. 3. Representative DSC thermograms of (a) unprocessed, (b) spray-dried and (c) crystallised lysozyme stored for 20 weeks at: (A) 4 °C/2% RH and (B) 40 °C/75% RH. Samples heated from 25 to 275 °C, at 10 °C/min.

20 °C/65% RH, 30 °C/65% RH and 40 °C/75% RH and for spray-dried form reserved at 40 °C/75% RH) in the apparent denaturation temperature (T_m, the temperature at the peak maximum, at ∼200 °C) with increasing hydration from 2 to 75% RH for all samples compared with fresh samples. This implies higher thermal stability but this finding is contrary to the activity results and thus may be an artefact of the DSC scan experimental conditions (e.g. pan type) in which differences in the strength and extent of water binding may affect apparent thermal behaviour. For material stored at 60 °C/7% RH for 20 weeks (Table 3), unprocessed lysozyme showed a split denaturation endotherm at ∼200–210 °C (data not shown), displaying different thermal behaviour from the starting material. Likewise, a notable change in the thermal behaviour of the spray-dried particles was observed, in that the DSC scan showed no transition, indicating loss of thermal stability. Lysozyme crystals continued to exhibit a single denaturation endotherm (at ∼193 °C). The lower stability of the spray-dried lysozyme is a similar finding to the instability of spray-dried trypsinogen in the absence of excipients reported by Tzannis and Prestrelski (1999). At all conditions, enthalpy calculations provided no further clarification compared with T_m results, so enthalpies are not listed here.

From the preceding results, storage at 60 °C/7% RH (Table 3) showed the detrimental consequence of storage at 60 °C, especially on spray-dried protein. This illustrates why additives should generally be added during preparation of dried proteins, to prevent or decrease thermal stress. The results indicate that crystals maintained thermal stability (due to water in their crystal lattice that maintained crystals in a native folded state) better than did spray-dried lysozyme.

3.4. FT-Raman spectroscopy

The secondary structure of a protein contributes to its conformation. FT-Raman spectroscopy characterised the conformational stability of lysozyme samples, in the solid state and solution, during and after storage. Secondary structures were examined by comparison of FT-Raman spectra at regions: amide I (at ∼1660 cm^{-1}) for α-helix (Susi and Byler, 1986) and amide III (1250–1350 cm^{-1}) which are both characteristic of proteins (Quinn et al., 1999). We selected the band at ∼1255 cm^{-1} to represent the amide III region.

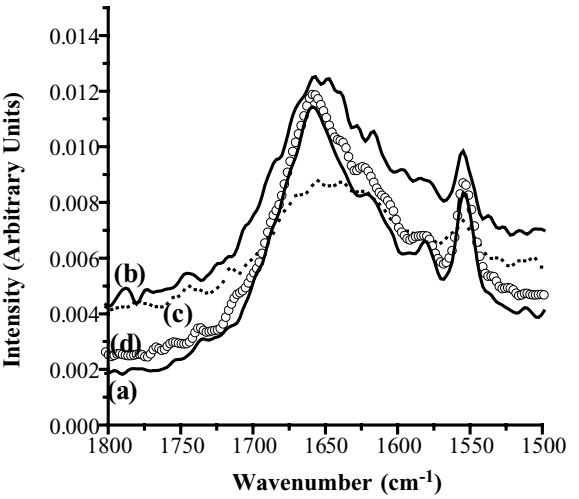

Fig. 4. Aqueous state FT-Raman spectra of: (a, solid line) fresh lysozyme (control), (b, solid line) unprocessed lysozyme after storage at 40 °C/75% RH for 20 weeks, (c, dotted line) spray-dried lysozyme at 40 °C/75% RH for 20 weeks and (d, open circles) crystallised lysozyme at 40 °C/75% RH for 20 weeks.

Spectra for aqueous solutions of unprocessed and processed samples pre- and post-storage were analysed after subtraction of water background to obtain protein Raman bands without interference by the water spectrum. The FT-Raman spectrum of lysozyme in aqueous solution (Fig. 4a, control spectrum) is similar to published data (Quinn et al., 1999). The plot shows the characteristic bands of lysozyme that exhibit α-helical structure with the amide I band at 1660 cm^{-1}; this band was mainly exploited to compare different spectra in water, for samples kept at different RHs and temperatures. Spectra of all samples stored at 4 °C for 20 weeks were identical to that of fresh lysozyme (control spectrum), i.e. they maintained the native structure after reconstitution. After storage at 20 °C/65% RH and 30 °C/65% RH for 20 weeks, unprocessed and crystallised protein provided spectra similar to that of the control. The spray-dried protein plot revealed some perturbations under these conditions—appearance of a new band at ∼1715 cm^{-1}, due to aggregation (see later), and shifting of amide I by approximately +9 cm^{-1}.

At 40 °C/75% RH after 20 weeks, the unprocessed sample (Fig. 4b) changed compared to control native

spectrum (splitting and shifting of amide I and appearance of a new band at $\sim$1788 cm^{-1}). The spray-dried spectrum (Fig. 4c) altered as well (splitting, shifting, loss of peak definition of amide I band and appearance of a new band at $\sim$1715 cm^{-1}). The appearance of bands at 1715 cm^{-1} indicated the presence of aggregates, as the peak in this position (above 1700 cm^{-1}) is rarely seen for proteins and indicates physical instability and aggregation (Souillac et al., 2002). The spectrum of lysozyme crystals showed least change on storage at this condition, in that there was a shift in amide I region approximately -3 cm^{-1} (Fig. 4d), implying the relative conformational stability of protein crystals compared with unprocessed and spray-dried forms. These findings were in good agreement with the results of activity measurements (see above).

Also in agreement with activity results, analysis of the spectra from reconstituted solutions of the solids stored at 60 °C/7% RH for 20 weeks indicated that temperature more detrimentally affected the spray-dried form than it did unprocessed material and crystals. The spray-dried spectrum (not shown) exhibited approximately $+4$ cm^{-1} shift in amide I and approximately $+8$ cm^{-1} change in amide III compared with control. Once again, we find that FT-Raman is a useful technique and supported the results of activity measurements (cf. Table 1).

Interestingly, for material reserved at different RHs and temperatures, the solid-state spectra of unprocessed and crystallised protein showed that these samples largely maintained structural stability as bands neither shifted nor decreased in intensity. In all cases, the amide I band was present at 1660 cm^{-1} with amide III at 1255 cm^{-1}. This feature would usually be interpreted as indicating that the secondary structure was maintained over 20 weeks, and thus is not as stability indicating as the solution obtained spectra. However, for spray-dried lysozyme, the fresh sample and those kept at 4 °C/2% RH for 4, 8 and 12 weeks revealed appreciable shifts of approximately $+4$ cm^{-1} and decreased intensity of amide III region. Peak shifts of a band at 1335–1342 cm^{-1} and a small shift of approximately -2 cm^{-1} in the amide I band also indicated perturbation of the secondary structure. Notably, for spray-dried samples at 20 °C/65% RH, 30 °C/65% RH and 40 °C/75% RH, the two characteristic bands moved toward their more usual position with time. After 20 weeks, the peak positions of amide I and amide III were at $\sim$1660 and 1255 cm^{-1}, respectively. This suggests that as the spray-dried sample took up moisture, some renaturation of the secondary structure occurred. The loss of activity that results under such storage conditions may have been due to aggregation and chemical decomposition.

Regarding storage at 60 °C/7% RH for 20 weeks, FT-Raman solid-state spectra of unprocessed and crystallised lysozyme revealed shifts in amide III band ($\sim$2 cm^{-1}). However, spray-dried protein underwent marked drift in amide III (approximately $+7$ cm^{-1}) and showed decreased intensity of amide I, illustrating the decreased conformational stability of the spray-dried form. From these data, it was apparent that spray drying of the protein substantially altered the structure and stability of lysozyme compared with unprocessed and crystallised protein. Costantino et al. (1994) suggested that such change arises from the increasing effect of dehydration with time.

3.5. High sensitivity differential scanning calorimetry (HSDSC)

HSDSC provides information about protein folding and stability by measuring those thermodynamic parameters that control protein folding–unfolding transitions (Cooper and Johnson, 1994). Accordingly, HSDSC was utilised to assess the thermal stability of lysozyme solution after dry storage. Fig. 5A and B displays the HSDSC profiles for thermal denaturation of lysozyme samples kept at 4 °C/2% RH and 40 °C/75% RH for 20 weeks and Table 4 summarises the denaturation temperatures (mid-point of the transition peak).

Table 4
Denaturation temperature (T_m) of lysozyme after storage for 20 weeks; high sensitivity differential scanning calorimetry (HSDSC)

Conditions		T_m (°C)		
°C	% RH	Unprocessed	Crystallised	Spray-dried
Fresh		76.1 (0.06)	76.3 (0.11)	76.2 (0.06)
4	2	76.2 (0.08)	76.0 (0.15)	76.3 (0.13)
20	65	76.1 (0.12)	76.0 (0.16)	75.6** (0.08)
30	65	76.1 (0.09)	76.1 (0.08)	75.6** (0.07)
40	75	75.7** (0.04)	75.9* (0.11)	75.1** (0.03)
60	7	75.5** (0.05)	75.6** (0.06)	75.3** (0.04)

Values within parenthesis are S.D., $n = 3$.
* $P < 0.05$ compared to fresh sample.
** $P < 0.001$ compared to fresh sample.

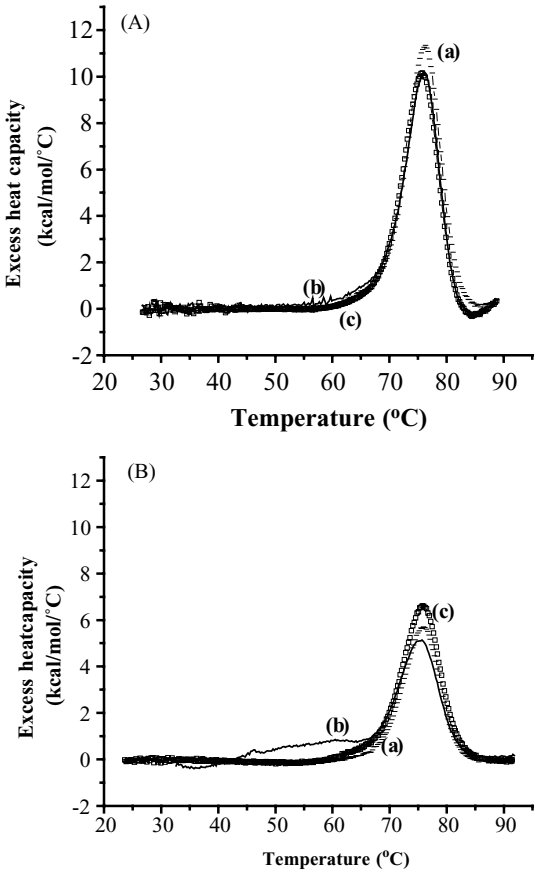

Fig. 5. Examples of normalised calorimetric data from high sensitivity differential scanning calorimetry (HSDSC) for the thermal denaturation of (a, open bar) unprocessed, (b, solid line) spray-dried and (c, hollow square) crystallised lysozyme after 20 weeks at: (A) 4 °C/2% RH, (B) 40 °C/75% RH. Heated from 20 to 90 °C, at 1 °C/min.

From Table 4, it is apparent that the transition temperatures (T_m) of unprocessed and crystallised protein samples maintained at 20 °C/65% RH and 30 °C/65% RH were similar to those before storage and those kept at 4 °C/2% RH (Fig. 5Aa and c), demonstrating their thermal stability. With spray-dried material kept at 20 °C/65% RH and 30 °C/65% RH, HSDSC data revealed appreciable changes in the melting characteristics compared to fresh spray-dried material and samples reserved at 4 °C/2% RH (Fig. 5Ab). There was a significant decrease ($P < 0.001$) in T_m, revealing a lowered thermal stability.

For storage at 40 °C/75% RH (Fig. 5B, Table 4), T_m was lower ($P < 0.001$) for spray-dried and unprocessed samples, confirming lower thermal stability. In the case of spray-dried sample, the base line deviated at lower temperatures (Fig. 5Bb) which may have been due to misfolded regions. Anekwe (1999) observed similar deviations for lysozyme after rescan. Notably, and in good agreement with activity data, for crystallised lysozyme, the decrease in T_m was less significant ($P < 0.05$). Again in agreement with activity data, on storage at 60 °C/7% RH after 20 weeks, all samples revealed a decreased T_m ($P < 0.001$).

3.6. Long-term stability study

The results of the accelerated stability study were confirmed by the results of a longer-term bench-top stability study. The previously discussed less-sensitive solid-state Raman technique identified no changes in peak position for crystalline lysozyme after 2 years ambient storage, only minor changes for the unprocessed sample (amide I (approximately $-1\,cm^{-1}$) and in amide III (approximately $+2\,cm^{-1}$)), but was sufficiently sensitive to identify more major changes for the spray-dried sample. FT-Raman spectra of spray-dried material indicated alteration of the secondary structure of this form, as there were: decrease in intensities of all bands; shifts in amide I (approximately $+5\,cm^{-1}$) and in amide III (approximately $-3\,cm^{-1}$) and splitting of the band at $1335\,cm^{-1}$. A similar conclusion was arrived at from the results of HSDSC analysis of the 2-year ambient stored batches. HSDSC data revealed that crystallised lysozyme maintained its thermal stability after storage, as there was no decrease in the transition temperature ($T_m = 76.4$ °C after 2 years) compared with fresh crystals. In contrast, unprocessed and spray-dried samples showed marked decrease in T_m ($T_m = 75.4$ and 73.5 °C for unprocessed and spray-dried lysozyme, respectively), indicating reduced thermal stability of these samples after shelf storage.

4. General discussion and concluding remarks

Overall results suggested that after storage lysozyme crystals are more stable and active compared to unprocessed and spray-dried protein. Spec-

troscopic and thermal analysis data supported the findings of activity studies. These crystals maintain thermal stability in both solid state (as shown in DSC) and thus have better functionality in solution (as indicated by HSDSC). Their conformational integrity was most stable (as revealed by FT-Raman spectroscopy). Such intactness was indicated as essential for enzymatic activity (confirmed by biological assay). Crystals differ from a spray-dried form when kept at 40 °C/75% RH for 20 weeks. Spray drying of lysozyme perturbs melting characteristics after storage (see HSDSC data) and alters protein secondary structure (FT-Raman results). Accordingly, the biological activity of spray-dried samples decreases. Based on TGA and FT-Raman studies, we conclude that spray-dried samples stored at 40 °C/75% RH absorb more water than crystals and this entrapped water interacts with the protein, changing the native structure. Also, this storage condition increased the water content of crystals; but as the amount of water adsorbed at the solid–air interface depends on the available surface area, the water content is low. In addition, the internal domains of crystals remain intact due to their rigidity. Our results were consistent with an observation that preparation of proteins using drying techniques usually requires addition of stabilising additives to decrease the deleterious effect of thermal stress and to replace the stabilising effect of water. Thus, our study suggests that lysozyme crystals are more stable and active, compared to unprocessed and spray-dried forms, without added excipient. Thus, crystals are carrier free, so they can provide high doses at the delivery sites (St. Clair et al., 1999). Accordingly, a crystallisation technique can provide stable and effective protein formulations.

The overall finding that crystalline proteins are more stable than an amorphous form was also observed by Shenoy et al. (2001). They reported that incubation of amorphous glucose oxidase at elevated temperature (50 °C) profoundly and irreversibly changed the protein structure. This led to aggregation and loss of activity, while the protein maintained its native structure when in a crystalline form. The results highlight once again the importance of storage conditions and physical form on the stability of biological macromolecules. From these data, we concur with Gross (2001) that "It is about time that we began to make use of protein crystals" and exploit a formulation advantage.

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