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International Journal of Pharmaceutics 247 (2002) 79–90

international journal of pharmaceutics

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Integrity of crystalline lysozyme exceeds that of a spray-dried form

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Received 5 March 2002; received in revised form 8 July 2002; accepted 10 July 2002

Abstract

The development of proteins as therapeutic agents is challenging partly due to their inherent instabilities. Consequently, crystallisation and spray drying techniques were assessed to determine their effects on protein integrity using lysozyme as a model protein. Unprocessed, crystallised and spray-dried lysozyme were characterised by: thermal analysis using hot stage microscopy (HSM), differential scanning calorimetry (DSC), high sensitivity differential scanning calorimetry (HSDSC) and thermogravimetry (TGA); and spectroscopic analysis employing Fourier transform Raman (FT-Raman). Moisture contents were determined by TGA and Karl Fisher titration (KFT). Enzymatic assay measured biological activity. HSM showed no changes in crystals until complete melting. TGA and KFT indicated that spray-dried lysozyme contained a lower moisture content than crystals, hence the higher apparent thermal stability was shown by DSC. HSDSC revealed that crystallisation and spray drying did not affect the denaturation temperature of lysozyme in solution when compared with unprocessed material. However, in the solid state, FT-Raman spectra showed perturbation of the conformational structure of spray-dried sample, whereas crystal conformation remained intact. Enzymatic assay revealed increased activity retention of crystals compared with spray-dried powder. Hence, crystals maintained the conformational integrity and activity of lysozyme in solution.

Keywords: Protein integrity; Thermal analysis; Spectroscopic analysis; Lysozyme crystals; Enzymatic assay; Spray-dried lysozyme

1. Introduction

Proteins have unique chemical and physical properties, posing difficult stability problems. Accordingly, stable formulations that maintain their native structure and functionality are needed to prepare proteins as pharmaceutical products. Moreover, the biological activity of a protein depends on its native structure as determined by the three-dimensional arrangement of amino acids at the active site (Rosenberger, 1996). Proteins can degrade via chemical instability (deamination, oxidation, proteolysis, disulphide exchange, racemisation and β-elimination) and physical change (denaturation, aggregation, precipitation and adsorption at interfaces) (Manning et al., 1989; Parkins and Lashmar, 2000). Approaches that inhibit these degradation processes increase pro-

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00379-4

tein conformational integrity and stability. Most literature cited the stabilisation of proteins by additives (Takeda et al., 1988; Taneja and Ahmed, 1994; Katakam et al., 1995; Kreilgaard et al., 1999; Patel et al., 2001).

This study focused on protein stabilisation by processing using spray drying and crystallisation techniques. Water interacts with protein, increasing flexibility and perturbation of conformational structure (Zaks, 1992). Hence, exclusion of water from material through drying may maintain integrity and stability (Skrabanja et al., 1994; Purie et al., 1996). During spray drying, proteins may thermally degrade, as heat is the main physical stress. However, molecules are in dilute solution and the water evaporates rapidly and thus exposure time to high temperature is very short (Mathias et al. 1991). The factors affecting product stability are the residual moisture content and its distribution.

Initial thoughts may lead one to generally assume that the amorphous state is preferred for proteins and that crystallisation is often damaging. This is true for amorphous protein and stabiliser combinations where crystallisation of the stabiliser can lead to shear stress of the protein. However, for pure protein systems, the amorphous state may facilitate a more mobile and flexible conformation, which could undergo chemical or physical degradation. Thus, since crystallisation can provide pure and rigid protein crystals, in general, crystalline drugs are stable towards chemical degradation as their environment provides an inert and rigid medium for the molecule that prevents the motion required for reactions to proceed (Pikal and Rigsbee, 1997). Literature describing the stabilisation of proteins by crystallisation is sparse (e.g. St. Clair et al., 1999).

The goals of this research were to investigate and compare the effects of spray drying and crystallisation on lysozyme-conformational stability, integrity and activity, and to evaluate the spray-dried and crystallised products employing physicochemical and biological analyses. Lysozyme was chosen as a model protein because it is commercially available and well characterised in the literature (Rosenberger, 1996).

2. Materials and methods

2.1. Materials

Chicken egg-white lysozyme (purity 95%), sodium chloride (99.5%), sodium phosphate (99.3%), Tween 80, and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Company (St. Louis, MO). Sodium acetate anhydrous (98%), potassium dihydrogen orthophosphate ($>99\%$) were obtained from BDH Chemicals Ltd., Poole, UK. Water was deionised and double distilled.

2.2. Preparation of spray-dried lysozyme

An aqueous lysozyme solution (1%, w/v) was dialysed using a membrane with a molecular weight cut off 13,000 Da (Medicell International Ltd.) and was spray-dried through a Büchi 190 Mini spray dryer. The feed solution passed from an atomising nozzle 0.5 mm diameter to the drying chamber via a peristaltic feed pump (Masterflex, Cole Parmer) at a flow rate of 4–5 ml/min, and was dried at an inlet temperature of 120 ± 2 °C, outlet temperature was 52 ± 3 °C. Cooling water circulated through a jacket around the nozzle to minimise protein degradation. Dried powder was collected by a cyclone separator and stored in vials at 4 °C.

2.3. Preparation of lysozyme crystals

Proteins crystallise in supersaturated solutions when the concentration exceeds equilibrium solubility. Accordingly, the concentration of the macromolecules and parameters that affect its solubility control the supersaturation. In this work, supersaturation was induced at a high lysozyme concentration by using sodium chloride as a precipitating agent. Also, 0.1 M sodium acetate buffer, pH 4.6 was employed as lysozyme solubility decreased with increasing acetate concentration from 0.01 to 0.1 M and increasing the acetate concentration beyond 0.1 M raised lysozyme solubility (Forsythe and Pusey, 1996). Lysozyme crystals formed as a result of a "salting-out" effect of NaCl on non-polar groups of the protein (Yoshioka et al., 1993). Lysozyme was crystallised

using three processes: batch, microbatch and vapour diffusion methods.

2.3.1. Batch crystallisation method

Lysozyme (4 g) was dissolved in 100 ml of 0.1 M sodium acetate buffer, pH 4.6, and 10 g of NaCl was dissolved in 100 ml of the same buffer. Equal volumes of lysozyme and NaCl solutions were passed through a 0.2 μm filter and mixed to produce a solution having 2% lysozyme and 5% NaCl. The sealed solution was stored at 20±1 °C for 14 days. Crystals formed were recovered by filtration and dried.

2.3.2. Microbatch crystallisation method

Droplets (around 8 μl, using Gilson 20 μl pipette) of 2% lysozyme and 5% NaCl solution in 0.1 M sodium acetate buffer, pH 4.6, were immersed in paraffin oil and stored in vials at 20±1 °C for 14 days. Crystals formed in aqueous droplets as the oil acted as an inert sealant and prevented evaporation of protein solution. Consequently, slower supersaturation and growth rates tended to produce better, but fewer, crystals (Blagova and Kuranova, 1999).

2.3.3. Vapour diffusion method

To a reservoir was added 20 ml of 4% NaCl solution in 0.1 M sodium acetate buffer, pH 4.6. Droplets (15 μl) from a mixture of 2% lysozyme and 3% NaCl in the same buffer were transferred to a siliconised cover and the covered reservoir was kept at 20±1 °C. The different concentrations of the precipitant in the reservoir and the protein-containing droplets caused water to diffuse from the protein droplet to the reservoir solution until equilibrium was reached. Therefore, the concentration of the protein increased and it crystallised (Chayen, 1999). Crystals were formed after about 10 days.

2.4. Preparation of spray-dried and crystallised lysozyme containing Tween 80

The above procedures for the preparation of spray-dried and crystallised lysozyme, using the batch method, were employed. Tween 80 (at 0.09%, w/v) was added to distilled water and 0.1 M sodium acetate buffer, pH 4.6, for spray drying and crystallisation, respectively.

2.5. Microscopy of crystals

Microscopy is valuable for studying crystal shape as it provides quick and simple information. A Nikon Labophot-2 microscope (Nikon, JVC, Japan) at 100-fold magnification identified the possible crystalline forms of lysozyme prepared by the above methods.

2.6. Thermal analysis

2.6.1. Hot stage microscopy

Hot stage microscopy (HSM) determined the melting range of unprocessed, spray-dried and crystallised lysozyme and detected changes of shape and birefringence of samples. Visual appearances of thermal events were recorded using a Nikon Labophot-2 optical polarising microscope (Nikon, JVC, Japan) connected to a hot stage unit (Stanton Redcroft, UK), 109 digital display unit (Colorado video, Inc., USA), and a Mitsubishi HS-S5600 double density video recorder. The copies were produced through the Mitsubishi colour video copy processor. Samples were heated (i) from 25 to 250 °C at 5 °C/min and (ii) from 25 to 150 °C at 10 °C/min, then from 150 to 250 °C at 1 °C/min. Changes in sample shapes were monitored in triplicate.

2.6.2. Differential scanning calorimetry

A Perkin Elmer differential scanning calorimeter 7 (DSC 7, Perkin Elmer Ltd., Beaconsfield, Bucks) analysed materials in triplicate. Solid samples (3–7 mg) sealed in aluminium differential scanning calorimetry (DSC) pans with vented lids and loaded in sample cells under nitrogen, were scanned from 25 to 275 °C at 10 °C/min.

2.6.3. Thermogravimetric analysis

Thermogravimetry (TGA) was used with DSC for assignment of thermal events, as decomposition reactions coincide with weight changes (McCauley and Brittain, 1995); it was also employed to determine the moisture contents of lysozyme samples. Analysis used a TGA 7 (Perkin

Elmer Ltd.). Solid samples (3–13 mg) analysed in triplicate were loaded on an open platinum TGA pan suspended from a microbalance and heated from 25 to 275 °C at 10 °C/min.

2.7. Determination of moisture content using Karl Fischer titration

Samples (in triplicate) were titrated using a 701 KF Titrino with 703 Ti stand (Metrohm Ltd., Switzerland) after calibration with a standard solution of water in methanol (5 mg water and 1 ml methanol).

2.8. Fourier transform Raman spectroscopy

The Fourier transform Raman (FT-Raman) spectra of solid samples were collected using a Bruker IFS 66 (Karlsruhe, Germany) spectrometer with FRA 106 FT-Raman module equipped with a Nd^{3+}:YAG laser emitting at a wavelength of 1064 nm. Material was analysed in stainless steel sample cups in triplicate; the laser power was approximately 200 mW for unprocessed samples and 328 mW for processed materials. Spectra were the average of 400 scans at 4 cm^{-1} resolution over the range, 3500–500 cm^{-1}, at 25±1 °C.

2.9. Characterisation of lysozyme samples in aqueous state

2.9.1. High sensitivity differential scanning calorimetry

Solution samples were analysed in triplicate with a Microcal MCS differential scanning calorimeter (Microcal Inc., MA). 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. Both sample and reference cells were filled completely to maintain equal volumes and the same amount of lysozyme in each run. The sample and reference were heated from 20 to 90 °C at 1 °C/min under 2 bar nitrogen pressure. A base line was run before each measurement by loading the reference in both the sample and reference cells; this base line was subtracted from the protein thermal data, and the excess heat capacity was normalised for lysozyme concentration using ORIGIN DSC data analysis software.

2.9.2. Biological activity assay

A bacterial suspension was prepared by adding 20 mg of *M. lysodeikticus* to 90 ml of phosphate buffer 0.067 M, pH 6.6, and 10 ml of 1% NaCl. The enzyme solutions (15 μg/ml) of unprocessed, spray-dried and crystallised lysozyme were prepared in the same buffer. The biological reaction was initiated by addition of 0.5 ml of each enzyme solution to 5 ml of the bacterial suspension. The decrease in the absorption rate at 450 nm was monitored (Remmele et al., 1990) using a UV–Vis spectrophotometer (Pu 8700, Philips, UK). Five assay replicates per sample were performed. The Student's *t*-test was used throughout as a test for significance.

3. Results and discussion

3.1. Microscopic examination of lysozyme crystals

Fig. 1 presents the microscopic shape of lysozyme crystals prepared by batch, microbatch and vapour diffusion crystallisation methods; lysozyme

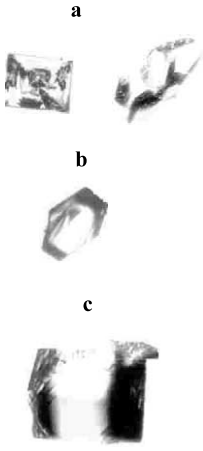

Fig. 1. Photomicrographs (magnification × 100) of tetragonal lysozyme crystals prepared by (a) batch method, (b) microbatch method and (c) vapour diffusion method.

crystals are tetragonal independent on the method of crystallisation.

The batch crystallisation method was the highest yielding technique; consequently, its product was used for physicochemical characterisation and analysis of lysozyme crystals.

3.2. Thermal analysis

3.2.1. HSM

The effects of temperature increase on unprocessed, spray-dried and crystallised lysozyme were studied via hot stage polarising microscopy at two heating rates to monitor protein stability. First, all samples were heated from 25 to 250 °C at 5 °C/min; shape changes are shown in Fig. 2. Fig. 2a reveals that for this sample the edges of unprocessed lysozyme particles started to melt first at ~234 °C, followed by internal melting at ~243 °C; hence particle shape changed with increasing temperature (Elkordy et al., 2001). The melting range was 231±4 to 245±5 °C. Fig. 2b indicates that the spray-dried sample was amorphous and non-birefringent as the particles were opaque and did not alter in shape on heating; consequently, the melting range of these particles was not recorded using HSM although decomposition was observed.

For crystals, Fig. 2c shows that lysozyme started to melt at ~222±6 °C, observed as internal turbidity without change in external shape until complete melting at ~230±4 °C. Moreover, increased temperature was accompanied by birefringence until ~230 °C, indicating high thermal stability of crystals. Interestingly, lysozyme crystals melted over a narrower melting temperature range (~8 °C) than that of unprocessed protein (~14 °C), indicating that crystals were purer (Kuhnert and Brandstatter, 1971). This is also shown by the relatively narrow endotherm obtained from calorimetric data (see Section 3.5.1).

For the second heating cycle, the melting region of unprocessed and crystallised samples was determined over 150–250 °C at 1 °C/min to study the effect of a slower heating rate on the melting characteristics (Fig. 3). Fig. 3a reveals that unprocessed lysozyme carbonised without melting. However, lysozyme crystals melted at ~220±2 to

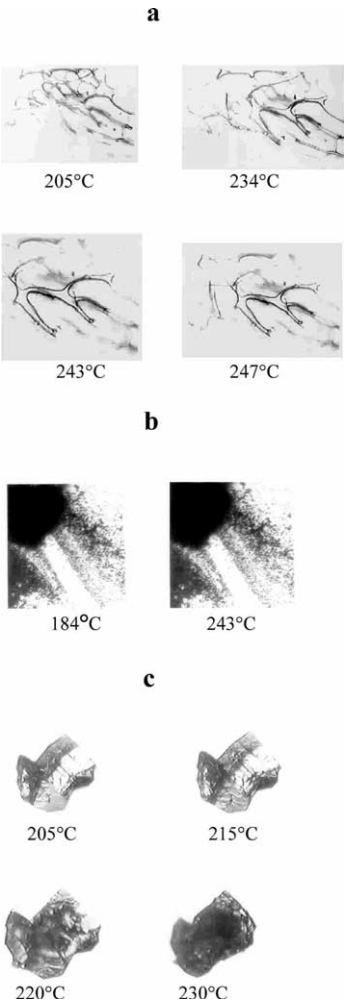

Fig. 2. HSM photomicrographs (magnification ×100) showing melting of (a) unprocessed, (b) spray-dried and (c) crystallised lysozyme. Conditions: heated from 25 to 250 °C, heating rate: 5 °C/min.

224±3 °C, suggesting strong binding of water molecules to the crystal structure, as the melting characteristics were unaffected by the slow heating rate.

3.2.2. DSC

Representative DSC curves of unprocessed and processed lysozyme are illustrated in Fig. 4. The curves are characterised by two endotherms, one being very broad at about 55–108 °C and a second endotherm of varying broadness at about

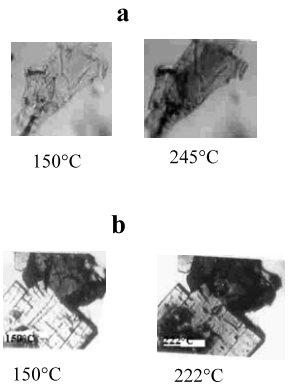

Fig. 3. HSM photomicrographs (magnification ×100) of melting of (a) unprocessed and (b) crystallised lysozyme. Conditions: heated from 150 to 250 °C; heating rate: 1 °C/min.

200 °C. Specifically, in the plots for lysozyme (unprocessed and processed), the endotherm at higher temperatures is thought to represent the denaturation transition with some decomposition and the peak maximum was considered to reflect the denaturation melting temperature (T_m). For unprocessed, spray-dried and crystallised lysozyme, T_m-values were 200.3 ± 0.4, 205.9 ± 0.8 and 195.3 ± 0.3 °C, while the calorimetric enthalpy changes (the areas under the transition peak, ΔH_{cal}) were 20 ± 4, 13 ± 3 and 20 ± 5 kcal mol^{-1}, respectively. Whilst the T_m of spray-dried lysozyme was statistically significantly higher ($P < 0.05$) than that of the unprocessed form and likewise the T_m of lysozyme crystals was significantly less ($P < 0.05$) than that of unprocessed particles, it is probable that the varying degrees of decomposition as observed from HSM studies do not allow a direct rank-order stability assignment. Furthermore, differences in the strength and extent of water binding to the protein may affect thermal behaviour. For example, raised water content in a crystal lattice would act to increase the molecular mobility of protein (Tzannis and Prestrelski, 1999). In addition, unprocessed and crystallised lysozyme had similar mean denaturation enthalpies (ΔH_{cal}) which, whilst higher than that of the spray-dried material, were not statistically significantly so ($P > 0.05$). The broad endotherms which are due to water loss may also obscure the glass transition event and temperature (T_g), which is the temperature at which a material transforms from a stiff highly viscous glass to a viscoelastic rubber (Bell et al., 1995); for lysozyme $T_g \sim 50$–60 °C (Remmele et al., 1997).

It is known that the T_g for globular proteins such as lysozyme is difficult to determine by traditional DSC in the absence of excipients, because of the large internal heterogeneity of protein molecules, and existence of an extremely broad distribution of relaxation times (Fan et al.,

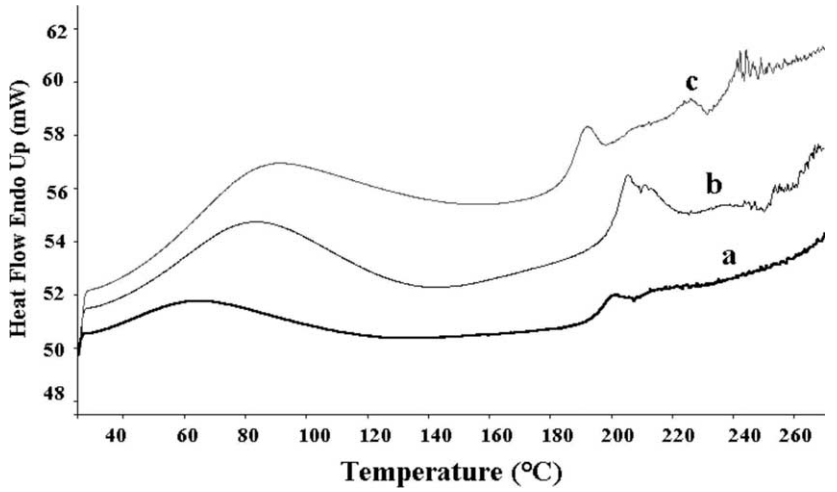

Fig. 4. Representative DSC thermograms of (a) unprocessed, (b) spray-dried and (c) crystallised lysozyme. Conditions: heated from 25 to 275 °C; heating rate: 10 °C/min.

1994; Green et al., 1994; Sarciaux and Hageman, 1997). Tween 80 was used as an excipient during the preparation of spray-dried and crystallised lysozyme, to try to shift T_g to a lower temperature below the broad endotherm and to reduce associated water content. Accordingly, the measured T_g will be the T_g of the protein/excipient system, as glass transition theory proposes that a mixture of two substances with different T_g-values will produce a compound with an intermediate T_g (Bell et al., 1995).

The DSC traces of preparations containing Tween 80 are shown in Fig. 5. Whilst unfortunately no clear T_g event is observable in the lower temperature endotherm, the higher temperature endotherm is noticeably much reduced. The mechanism of the effect of the surfactant requires further investigation.

3.2.3. TGA

TGA curves are presented in Fig. 6 with quantitative data in Table 1. Each trace is broadly characterised by three stages of weight loss. In the first period, the fall is attributed to loss of adsorbed surface water as evident by weight changes up to the transition temperature of lysozyme (this was confirmed by DSC where broad endotherms were apparent over the same temperature range as the TGA weight loss). In the second stage, the weight decreases slightly. In the third section, weight loss is ascribed to decomposition as it occurred at the melting temperature. This is reasonable as there were no significant differences in the weight losses in the third stage, which were 3.20 ± 0.3, 3.16 ± 0.2 and $2.93 \pm 0.3\%$ (w/w) for unprocessed, spray-dried and crystallised samples, respectively. This suggests that the three samples underwent the same reaction mechanism.

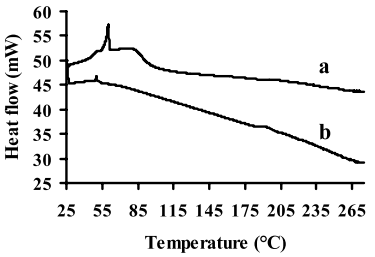

Fig. 5. Example DSC thermograms of (a) spray-dried and (b) crystallised lysozyme containing 0.09% Tween 80. Conditions: heated from 25 to 275 °C; heating rate: 10 °C/min.

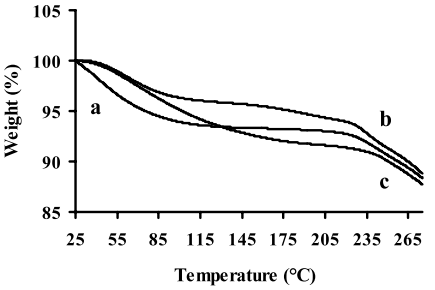

Fig. 6. Example TGA thermograms of (a) unprocessed, (b) spray-dried and (c) crystallised lysozyme. Conditions: heated from 25 to 275 °C; heating rate: 10 °C/min.

Table 1
Weight loss of lysozyme determined by thermogravimetric analysis upon heating from 25 to 275 °C at 10 °C/min[a]

Process	Stages of weight loss	
Unprocessed		
	1	6.55 (0.4)
	2	1.70 (0.3)
	3	3.20 (0.3)
Spray-dried		
	1	4.19 (0.2)
	2	2.89 (0.1)
	3	3.16 (0.2)
Crystallised		
	1	7.30 (0.1)
	2	2.02 (0.3)
	3	2.93 (0.3)

[a] Values between brackets are S.D.

Table 1 shows that the water content of commercial protein as measured over the two first stages ($8.25 \pm 0.7\%$, w/w) was similar ($P > 0.05$) to that of the processed protein (7.08 ± 0.3 and $9.32 \pm 0.4\%$ (w/w) for spray-dried and crystallised lysozyme). However, the water content of crystals was significantly higher ($P \leq 0.001$) than that of spray-dried powder. With regard to the ease with which water could be removed from the various lysozyme samples, water associated with the crystals was the most firmly bound since its initial water loss occurred at higher temperatures. It seems probable

that this strength of association may have contributed to the lower transition temperature (T_m) observed for crystals. This interpretation is consistent with the data of Bell et al. (1995), who found that increasing the moisture contents of lyophilised bovine somatotropin and lysozyme decreased their thermal stabilities. Hence, whilst the T_m of solid-state crystals (highest water content) was lower than that of solid-state spray-dried lysozyme (lowest water content), strength of association as well as magnitude of water content is a factor impacting on protein thermal stability.

3.3. Determination of moisture content using Karl Fisher titration

Moisture contents as determined by Karl Fisher titration (KFT) were 8.4 ± 0.7, 7.3 ± 0.2 and $9.5 \pm 0.8\%$ (w/w) for unprocessed, spray-dried and crystallised samples, respectively. These results were similar and consistent with the moisture content results obtained from first and second stages of TGA thermograms (Table 1). Thus, KFT confirmed the TGA results.

3.4. FT-Raman spectroscopy

Infrared (IR) spectroscopy is a well-used tool for studying the molecular characterisation of pharmaceutical products. However, Raman spectroscopy offers an alternative method for investigating the molecular state of protein systems (Quinn et al., 1999), as IR and Raman vibrational analyses give similar but complementary data (Bugay and Williams, 1995). The secondary structures of unprocessed and processed lysozyme were examined by comparison of FT-Raman spectra at amide I and III regions that are characteristic of proteins. The amide I frequency depends on the local conformation of the amide group, whether it is hydrogen-bonded to water or another amide, and on the coupling effects between residues (Winters et al., 1996). Hence, the amide I band shape and position provide information about protein conformational structure. Fig. 7a and b displays the spectra of unprocessed, spray-dried and crystallised solid samples over the range 1700–1500 cm^{-1} (amide I) and 1400–1250 cm^{-1} (amide III). Important peak positions are in Table 2. Fig. 7a and b shows that unprocessed lysozyme is predominantly α-helical in structure as it has a strong amide I band at ~ 1660 cm^{-1}; this is in complete agreement with previously published spectra (Winters et al., 1996; Quinn et al., 1999).

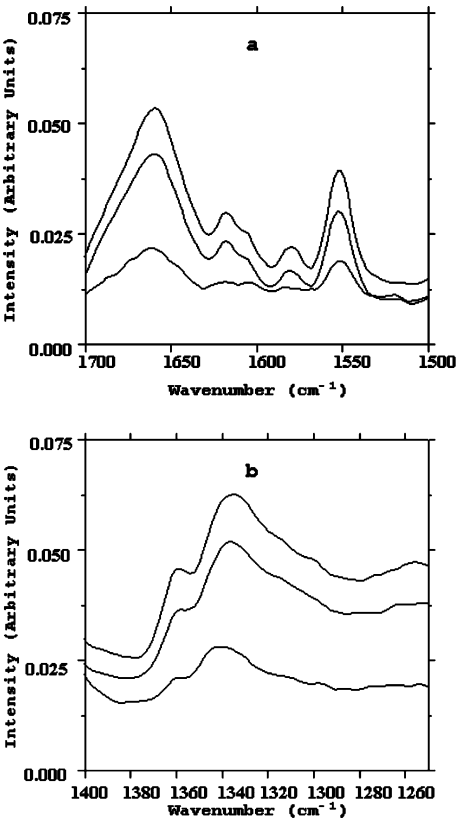

Fig. 7. FT-Raman spectra of crystallised (upper trace), unprocessed (middle trace) and spray-dried (bottom trace) lysozyme: (a) 1700–1500 cm^{-1} (amide I) and (b) 1400–1250 cm^{-1} (amide III).

Table 2
FT-Raman peaks (cm^{-1}) of amide I and III bands for unprocessed and processed lysozyme[a]

Process	Amide I	Amide III
Unprocessed	1660	1336
Spray-dried	1662	1341
Crystallised	1660	1335

[a] Data are wavenumber positions of bands obtained from Fig. 7a and b.

Also, the amide III band was at $\sim 1336\ cm^{-1}$ (Costantino et al., 1995).

Following spray drying of lysozyme, the spectrum shifted slightly ($\sim 2\ cm^{-1}$) in amide I band (Table 2); moreover, the shape of this band changed and appeared as two overlapping peaks (Fig. 7a, bottom trace). The spray-dried spectrum at amide III region (Fig. 7b, bottom trace) shifted markedly from 1336 to 1341 cm^{-1} (Table 2) compared with commercial protein. Accordingly, spray drying deteriorated the secondary structure of lysozyme.

The spectrum of crystallised lysozyme is identical to that of unprocessed protein (Fig. 7a and b). This indicates that crystals maintain the conformational integrity of the protein.

3.5. Characterisation of lysozyme samples in aqueous state

3.5.1. High sensitivity differential scanning calorimetry

High sensitivity differential scanning calorimetry (HSDSC) has been used to study the thermal transition of biological macromolecules in dilute solutions and it has been employed in the formulation and stabilisation of protein drugs (e.g. Fatouros et al., 1997). Accordingly, we exploited HSDSC to evaluate the effects of spray drying and crystallisation techniques on protein stabilisation in solution.

Fig. 8 presents HSDSC traces for the thermal denaturation of unprocessed and processed lysozyme. Thermodynamic and kinetic parameters are in Table 3. HSDSC profile of unprocessed lysozyme (Fig. 8a) shows a single endothermic signal and the protein starting to unfold at ~ 65 °C with a mean peak T_m of 76.1 °C. The pre-transition region indicates a gradual change of the native structure with increasing temperature, while the post-transition region corresponds to chemical changes of the denatured state (Privalov and Khechinashvili, 1974). Fig. 8b and c indicates that spray-dried and crystallised lysozyme thermograms also have single unfolding endothermic transition peaks from the native state to the denatured state, revealing that lysozyme in solution from spray-dried and crystallised forms follows the two-state transition model typical for lysozyme (Freire, 1995).

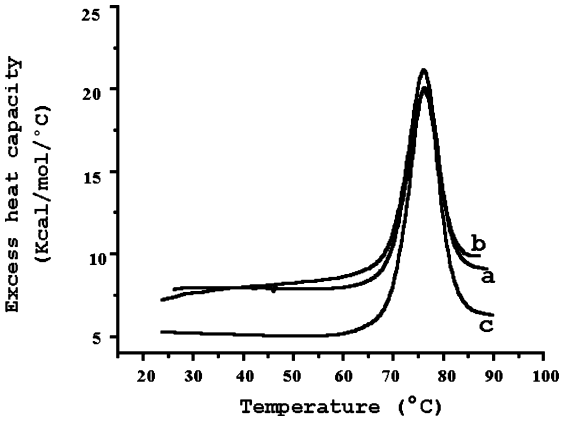

Fig. 8. Examples of normalised calorimetric data for the thermal denaturation of (a) unprocessed, (b) spray-dried and (c) crystallised lysozyme.

Table 3
Denaturation temperature (T_m), calorimetric enthalpy change (ΔH_{cal}), and temperature at half peak height ($\Delta T_{1/2}$) of unprocessed, spray-dried and crystallised lysozyme in solution[a]

Process	T_m (°C)	ΔH_{cal} (kcal mol^{-1})	$\Delta T_{1/2}$ (°C)
Unprocessed	76.1 (0.15)	73 (4.0)	6.2 (0.14)
Spray-dried	76.2 (0.02)	80 (3.2)	6.2 (0.23)
Crystallised	76.3 (0.12)	71 (4.6)	5.4 (0.23)

[a] Values between brackets are S.D. ($n = 3$). Conditions: 5 mg/ml lysozyme, 0.1 M sodium acetate buffer, pH 4.6, heating rate 1 °C/min.

Moreover, Fig. 8 and Table 3 indicate that spray drying and crystallisation did not affect significantly ($P > 0.05$) the T_m of unprocessed lysozyme. Thus the two formulations maintained the conformational stability of the enzyme after reconstitution as the transition temperature does not decrease. Also, the calorimetric enthalpy change (ΔH_{cal}) of spray-dried material is marginally higher ($P = 0.05$) than those of unprocessed and crystallised lysozyme, indicating some changes of the tertiary structure due to the larger heat absorption by spray-dried lysozyme.

The half width of the thermal denaturation peak ($\Delta T_{1/2}$) measures purity and homogeneity (cooperativity of the process) in the thermal stability

of protein molecules. The thermal stability of each protein molecule depends on the conformation of the molecule in the solution. Since such conformation is not rigid, some molecules may be structurally in labile states and easy to denature, and others may be in more stable states. Accordingly, protein thermally denatures over a broad temperature range. In other words, if the conformation of molecules in the solution is the same, the protein would denature at the same temperature and the denaturation peak would be sharp. From Table 3, it is apparent that $\Delta T_{1/2}$ of unprocessed protein was the same as that of the spray-dried form, but differed significantly from that of the crystallised lysozyme. The significant decrease in the $\Delta T_{1/2}$ for the crystal (higher co-operativity of the transition compared with unprocessed and spray-dried form) indicates the purity of crystals and tightening or restraint in conformation of lysozyme molecules in the crystal structure (Igarashi et al., 1999).

3.5.2. Biological activity assay

The biological activity of the reconstituted spray-dried and crystallised lysozyme was expressed as a percentage $\pm$S.D. relative to the aqueous solution of commercial lysozyme. The results were 84.9 ± 4.1 and $96.7 \pm 3.4\%$ for spray-dried and crystallised lysozyme, respectively. These results indicate that lysozyme crystals retained a significant higher biological activity ($P < 0.05$) compared with spray-dried lysozyme, demonstrating the impact of preparative technique on biological activity. The reduced activity, lower water content and the Raman spectral changes indicate that the dehydration of protein on spray drying perturbs the secondary structure and decreases the biological activity, which depends on the three-dimensional arrangement of amino acids at the active site. This reduction in activity emphasises the importance of water in maintaining the structural stability of globular proteins and in the thermodynamics of enzyme–substrate interactions (Shah and Ludescher, 1993; Allison et al., 1999). This was shown for crystals that maintained their biological activity due to water in crystal lattices. Also, this may be illustrated by the explanation of Nagendra et al. (1998), who proposed that the active site cleft is a heavily hydrated region in lysozyme molecules. Reduced activity upon dehydration appeared to arise from the removal of functionally important water molecules from the active site region and subsequent reduction in the size of this zone. A limitation of the use of lysozyme as a model protein is that because lysozyme is a stable protein and able to revert to its active state, it is possible that the solution studies do not reflect the conformational damage that may have occurred during solid processing.

4. Conclusions

The investigation demonstrated that lysozyme crystals maintained thermal stability, conformational integrity and activity better than the spray-dried form. This may be due to the physical process of protein crystallisation that has many analogies with protein folding. In both situations, hydrophobic forces play a dominant role and the concept of nucleation and growth applies to the formation of secondary or tertiary structures. Thus, the end result of the crystallisation and protein folding is a stable structure (Durbin and Feher, 1996). Consequently, a crystallisation technique can prepare pure, rigid and stable protein formulations. Also, the overall results suggest that removal of water by spray drying increases the apparent thermal stability in the solid state compared with unprocessed material, as illustrated by DSC data. Moreover, the spray-dried form showed the same T_m as unprocessed protein after reconstitution, as indicated by HSDSC. However, the calorimetric enthalpy change was slightly higher than that of unprocessed material, suggesting some perturbation in the native structure, as further evidenced by FT-Raman and biological analyses.

The water content within crystal lattices was higher compared with spray-dried powder as evident by TGA and KFT data. This water appears to be important in maintaining the native structure required for biological activity. Accordingly, lysozyme crystals were more stable and active in solution compared with spray-dried protein. Both spray drying and crystallisation

allow for directed control of particle properties more easily than a freeze drying process. The better stability of the crystals shows promise for the development of protein drug delivery systems.

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