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# Study of protein conformational stability and integrity using calorimetry and FT-Raman spectroscopy correlated with enzymatic activity

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## ABSTRACT

Maintaining protein conformational stability and integrity during formulation is critical for developing protein pharmaceuticals. Accordingly, high sensitivity differential scanning calorimetry (HSDSC) and Fourier transform (FT)-Raman spectroscopy were employed to assess conformational stabilities (thermal stability and folding reversibility) and structural integrities, respectively, for three model proteins: lysozyme, deoxyribonuclease I (DNase I) and lactate dehydrogenase (LDH) in lyophilised (as received) and spray-dried forms.

Enzymatic assay after cooling of thermally denatured protein solutions from HSDSC determined if thermal transition reversibility was related to biological activity. HSDSC data showed that molecules from lyophilised lysozyme were able to refold better than the spray-dried form. This was confirmed by enzymatic assay. Moreover, enzymatic assay results revealed that lysozyme folding reversibility was related to the native structure of the protein that is essential for the biological activity.

Thermal denaturation of DNase I and LDH samples in HSDSC was not reversible upon cooling of thermally denatured proteins (in contrast to lysozyme). Hence, it was decided to identify the effect of protein initial structures on its propensity to thermal denaturation via FT-Raman spectroscopy. In other words, proteins may denature with structural alterations due to stresses such as heat and the protein loses its enzymatic activity. Consequently, FT-Raman investigated the effects of spray drying and heating of solid DNase I and LDH samples, from differential scanning calorimetry, on protein conformational integrities. Lyophilised and spray-dried DNase I and LDH solid samples were heated to two temperatures, one before the apparent denaturation temperatures ( $T_m$ ) and the other after the  $T_m$ . Samples heated below their  $T_m$  showed some alterations of the secondary structure and some enzymatic activity. HSDSC and FT-Raman spectroscopy are useful techniques to study protein conformations and their results correlate with those of enzymatic activity.

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## 1. Introduction

Proteins fold in three dimensions. Protein structure is organised from primary structure to quaternary structure. The

primary structure or the amino acid sequence of the peptide chains is the ultimate determinant for the protein native form. The protein secondary structure is the assignment of  $\alpha$ -helices and  $\beta$ -sheets along the main peptide chain; whilst the ter-

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tiary structure is the assembly and interactions of the helices and sheets (Lesk, 2001). Consequently, there is a relationship between the protein secondary structure and its tertiary or native state; both structures mostly stabilised by non-covalent bonds. The protein quaternary structure presents only when the protein has more than one peptide chain. I agree with Lesk (2001) who has analysed a protein structure as a text. He described amino acids (primary structure) as letters; secondary structure as words; and tertiary structure as sentences. Hence, if a protein secondary structure perturbed; the biological activity of the protein will be affected because the stability of the protein secondary structure contributed to the stability of the protein native, tertiary state which is required for the protein biological activity. This will be shown in this study.

An important aspect in the preparation of proteins as pharmaceutical products is stabilisation of the native protein conformation (folded, three-dimensional, tertiary state). Moreover, it is not enough for protein conformation to be stable, but the protein must be able to find the state or folding pathway in a short time from a denatured, unfolded conformation (Lesk, 2001). Folding minimises exposure of non-polar groups and maximises exposure of polar groups to the solvent (Rupley et al., 1983). Accordingly, techniques to study protein conformational stability and integrity are of great importance. In this study, differential scanning microcalorimetry and FT-Raman spectroscopy were applied to study conformational stabilities and integrities of lysozyme, deoxyribonuclease I and lactate dehydrogenase (model proteins) in lyophilised and spray-dried forms.

Thermodynamic or conformational stability is defined as the difference in the free energy between the folded and unfolded state. This stability is the sum of weak non-covalent interactions including hydrogen bonds, van der Waal interactions, salt bridges and hydrophobic forces (Daniel, 1996). Differential scanning microcalorimetry can thermodynamically characterise protein unfolding transition by determining heat capacities, enthalpy changes,  $\Delta H$ , and melting temperatures,  $T_m$ , of native and denatured proteins (Matouschek et al., 1994; Takano et al., 1999). Furthermore, HSDSC was used to measure thermal transition reversibility that is no less important than  $T_m$  and  $\Delta H$  (Maneri et al., 1991; Boye et al., 1997; Tischenko et al., 1998); a protein transition is considered reversible if the molecule renatures upon cooling after heat treatment. Consecutive heating scans indicated the folding reversibility of thermal transitions (Remmele et al., 1998). The thermodynamic stability of proteins not only requires that the transition temperature ( $T_m$ ) and other thermodynamic parameters remain constant but also implies reversibility of protein from unfolded (denatured) to folded (native) state after removing the effect of an external condition such as heat. Anfinsen (1973) reported that denaturation of ribonuclease A, by heat or urea, was reversible when denatured molecules returned to a normal environment of temperature and solvent. Hence, both structure and enzymatic activity were regained as demonstrated in Anfinsen (1973).

Proteins are diverse molecules. Therefore, three model proteins, different in molecular weights and structures, were chosen for this study. Lysozyme, a globular protein, molecular weight 14.3 kDa, consists of a single 129-amino acid chain divided into two domains, one predominantly  $\alpha$  and the

other  $\beta$ , cross-linked by four disulfide bridges. The hydrophilic groups tend to concentrate on the surface and the hydrophobic groups in the core (Rosenberger, 1996). Deoxyribonuclease I (DNase I) which is a hydrophilic glycosylated protein, molecular weight 34 kDa, consisting of  $\beta$ -sheets more than  $\alpha$ -helix content.

Glycosylation has been reported to impact favourably the stability of some proteins (Wang et al., 1996) Accordingly, DNase I is structurally different from lysozyme. Lactate dehydrogenase (LDH) is a tetrameric protein, its structure described by Read et al. (2001). Its molecular weight is about 142 kDa. LDH is predominantly  $\alpha$ -helical structure with some  $\beta$ -sheets.

Proteins undergo various structural changes if physiological conditions alter. Accordingly, they may denature and the denatured protein may be different in the secondary structure or conformational integrity compared to that of the native protein. FT-Raman spectroscopy is a powerful tool for the characterisation of protein structure in diverse physicochemical environments (Pappas et al., 2000). For example, Quinn et al. (1999) studied lysozyme structural integrity in the hydrofluoroalkane propellants tetrafluoroethane and heptafluoropropane by FT-Raman spectroscopy; also Kudryavtsev et al. (2000) demonstrated the effect of ordering of internal water in thaumatin and lysozyme crystals by FT-Raman spectroscopy. In addition, unfolding of ribonuclease A in crystalline forms was evaluated by FT-Raman spectroscopy (Jacob et al., 1998). Elkordy et al. (2004) used FT-Raman spectroscopy to study the secondary structure of lysozyme samples in the solid state and solution during and after storage (stability studies).

In this study, FT-Raman spectroscopy evaluated conformational integrities of protein samples before and after spray drying in solid and aqueous states. Also, FT-Raman spectroscopy was exploited to detect protein denaturation in solid state after heat stress from DSC. In this research enzymatic assays determined (i) if refolding of denatured lyophilised (as received) and spray-dried lysozyme after thermal denaturation in HSDSC arises from the nativeness of the initial lysozyme structure. (ii) If changes in protein secondary structure (due to thermal effect) could affect the protein biological activity.

The goals of this study were to investigate the influence of spray drying and thermal stress on conformational stabilities and integrities of lysozyme, DNase I and LDH; and to study the relationship between protein folding reversibility and enzymatic activity.

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## 2. Materials and methods

### 2.1. Materials

Chicken egg white lysozyme in a lyophilised form (purity 95%), sodium phosphate (99.3%), deoxyribonucleic acid type I (DNA), calcium chloride (99%), 4-(2-hydroxyethyl)-1-piperazine-ethanolsulfonic acid (Hepes, 99.5%), sodium chloride (99.5%), magnesium chloride (98%), pyruvic acid (99+%, sodium salt), albumin from bovine serum (96%), standardised bovine pancreatic DNase I and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Company (St. Louis, MO). Deoxyribonuclease I in a lyophilised form (DNase I, isolated

from beef pancreas, activity 3230 Kunitz units/mg material) and lactate dehydrogenase in a lyophilised form (LDH, isolated from rabbit muscle, activity 331 U/mg material) were purchased from Biozyme Laboratories Ltd. (Gwent, UK). Sodium acetate anhydrous (98%), potassium dihydrogen orthophosphate (>99%) and nicotinamide-adenine dinucleotide (97%, reduced, disodium salt, NADH) were obtained from BDH Chemicals Ltd., Poole, UK. Water was deionised, double distilled.

## 2.2. Preparation of spray-dried proteins

Dialysed lysozyme, DNase I and LDH solutions were spray-dried in a Büchi 190 Mini Spray Dryer. This is a co-current two-fluid dryer in which the feed solution and atomising air pass separately to the atomising nozzle. The air was filtered through a 0.22 µm Milidisk filter (Millipore), heated to the required temperature, and moved to the spray-drying chamber (using compressed air at 2–6 bar) through an atomising nozzle (0.5 mm diameter). Silicone tubing of inner diameter 4 mm and a peristaltic feed pump (1–100 rpm, Masterflex, Cole Parmer) were used to feed the protein solutions to the nozzle and then to the drying chamber of the spray dryer. The flow rate was controlled by a flowmeter (Cole Parmer, 150 mm) and cooling water was circulated through a jacket around the nozzle to prevent protein degradation. The dried powder was separated from the air stream by a cyclone separator and the air was then removed from the system by an aspirator. The aspirator vacuum level was 10 (~30,000 l/h). The inlet and outlet temperatures were monitored with two PT-100 temperature sensors.

Table 1 summarises the conditions used for each protein. All dried protein powders were secured in vials below –15 °C in a desiccator over silica gel until assayed.

## 2.3. High sensitivity differential scanning calorimetry (HSDSC)

HSDSC was used to determine thermodynamic parameters and folding reversibilities of lysozyme, DNase I and LDH. HSDSC data were compared for low and high lysozyme concentrations.

Solution samples of lyophilised (as received) and spray-dried proteins were analysed with a Microcal MCS differential scanning calorimeter (Microcal Inc., MA, USA). Degassed samples (5 and 20 mg lysozyme/1 ml 0.1 M sodium acetate buffer, pH 4.6; 10 mg DNase I/1 ml 0.15 M NaCl with 100 mM CaCl<sub>2</sub>,

pH 6.3 and 5 mg LDH/1 ml 0.05 M potassium phosphate buffer, pH 7.4, this buffer was chosen as LDH provides a colourless solution in a concentration of 5 mg/ml as stated by the supplier) and reference (0.1 M sodium acetate buffer, pH 4.6 for lysozyme; 0.15 M NaCl with 100 mM CaCl<sub>2</sub>, pH 6.3 for DNase I and 0.05 M potassium phosphate buffer, pH 7.4 for LDH) were loaded into cells using a gas tight Hamilton 2.5 ml glass syringe. The samples and references were then heated from 20 to 90 °C at 1 °C/min for lysozyme; from 20 to 90 °C at 1.2 °C/min for DNase I (Chan et al., 1996) and from 20 to 70 °C at 1.2 °C/min for LDH (we have chosen this condition because LDH is very sensitive to heat and to the best of our knowledge, HSDSC data for LDH are not available in the literature). The folding reversibility of each protein denaturation was assessed by temperature cycling using the upscan–upscan method (UU) which employed two consecutive upscans, after the first upscan, the samples were immediately cooled in the calorimeter to 20 °C at 0.75 °C/min (the fastest cooling rate allowed by the instrument) and the heating cycle was immediately repeated. Transition reversibility was measured as ratio (%) of enthalpy change of second upscan ( $\Delta H_2$ ) over that of first upscan ( $\Delta H_1$ ). The calorimeter was temperature- and heat capacity-calibrated using sealed hydrocarbon standards of known melting points and electrical pulses of known power, respectively.

Additionally, HSDSC was utilised to heat DNase I samples in 5 mM Hepes buffer, pH 7.5, containing 1 mM CaCl<sub>2</sub> and 0.5 mM NaN<sub>3</sub> (6%, w/v) from 20 to 90 °C at 1.2 °C/min, then samples were cooled to 20 °C at 0.75 °C/min and analysed by FT-Raman spectroscopy (see below) to study the secondary structure of denatured DNase I solution. Hepes buffer, pH 7.5, containing 1 mM CaCl<sub>2</sub> and 0.5 mM NaN<sub>3</sub> (6%, w/v) was chosen after Bussian and Sander (1989).

Experiments were performed 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 protein concentration. Data analysis and deconvolution employed ORIGIN DSC data analysis software. The  $T_m$  (mid-point of the transition peak) and  $\Delta H$  (calorimetric enthalpy change, area under the peak) values for all transitions were calculated.

## 2.4. Differential scanning calorimetry (DSC)

A Perkin-Elmer differential scanning calorimeter 7 (DSC 7, Perkin Elmer Ltd., Beaconsfield, Bucks) examined DNase I and

**Table 1 – The spray-drying conditions applied to lysozyme, deoxyribonuclease I (DNase I) and lactate dehydrogenase (LDH)**

Protein	Concentration of protein solution (% w/v)	Inlet and outlet temperatures (°C)	Protein solution feed rate (ml/min)	The air spray flow rate (l/h)
Lysozyme	1 (in water)	120 ± 2, 52 ± 3	4–5	750 ± 50
DNase I	2 (in 0.15 M NaCl and 10 mM CaCl <sub>2</sub> ) <sup>a</sup>	90 ± 5, 55 ± 3	6	750 ± 50
LDH	1 (in water)	125 ± 5, 66 ± 4	8	750 ± 50

<sup>a</sup> DNase I is always contaminated with proteolytic materials such as chymotrypsinogen and trypsin. So, Ca<sup>2+</sup> was used to stabilise the protein against the proteolytic action (Price et al., 1969).

LDH. Solid samples (3–7 mg), sealed in aluminum DSC pans with vented lids and loaded in sample cells under nitrogen, were scanned from  $-30$  to  $210^{\circ}\text{C}$  at  $15^{\circ}\text{C}/\text{min}$  for DNase I and from  $25$  to  $250^{\circ}\text{C}$  at  $20^{\circ}\text{C}/\text{min}$  for LDH samples (this condition was chosen after Carpenter et al., 1993). Empty pans with vented lids were used as references. The calorimeter was temperature- and heat-calibrated with indium as a standard.

On the other hand, the DSC was employed to heat lyophilised (as received) and spray-dried DNase I and LDH solid states to two temperatures, one before the apparent denaturation temperature –  $T_m$  as determined by the DSC – and the other after the  $T_m$ . DNase I forms were heated from  $20$  to  $125^{\circ}\text{C}$  (before the  $T_m$  of DNase I, as determined by DSC) and from  $20$  to  $210^{\circ}\text{C}$  (above the  $T_m$ ) at  $15^{\circ}\text{C}/\text{min}$  and LDH samples were heated from  $25$  to  $150^{\circ}\text{C}$  (before the  $T_m$ ) and from  $25$  to  $250^{\circ}\text{C}$  (above the  $T_m$ ) at  $20^{\circ}\text{C}/\text{min}$ . Then, all samples were assayed for secondary structure, using FT-Raman spectroscopy (see below), and biological activity (see Section 2.6) to study the changes in conformational integrities and enzymatic activities of the proteins due to heat effect.

## 2.5. Thermogravimetric analysis

Thermogravimetric analysis determined the moisture contents of protein samples. Analysis used a TGA 7 (Perkin Elmer Ltd., Beaconsfield, Bucks). Solid protein samples (3–13 mg) were loaded on open platinum TGA pans suspended from a microbalance and the sample chamber was purged with dry nitrogen to avoid moisture from air. DNase I and LDH were scanned from  $25$  to  $210^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$  and from  $25$  to  $250^{\circ}\text{C}$  at  $20^{\circ}\text{C}/\text{min}$ , respectively.

## 2.6. FT-Raman spectroscopy

FT-Raman spectroscopy offers several advantages over infrared (IR) spectroscopy for analysis of peptides and proteins. Firstly, FT-Raman spectroscopy analyses do not require sample preparation when compared with IR. Secondly, FT-Raman spectroscopy studies are not hindered by variations in sample morphology. Under physiological conditions, the polypeptide chain of a protein is folded into a native state. The absorption of water at about  $1650\text{ cm}^{-1}$  occurs in the middle of the amide I band in the IR spectra, but because water is a weak FT-Raman scatter, hence the interference from water seen in IR is minimised. Consequently, FT-Raman spectroscopy can study proteins in their native form (aqueous state) besides their solid state and hence it was employed in our study.

The secondary structure of a protein can be determined by analysis of the amide band shape and position in FT-Raman spectra. The most sensitive modes to conformation in the FT-Raman spectrum are the amide I which arises from C=O stretching and amide III that mainly arises from the N–H bending ( $\delta\text{N-H}$ ). Proteins with  $\alpha$ -helical structure show a strong amide I band at about  $1645$ – $1660\text{ cm}^{-1}$ , whilst proteins with  $\beta$ -structure show an intense amide I band at about  $1660$ – $1670\text{ cm}^{-1}$  (Carey, 1982). The amide III band is at about  $1250$ – $1350\text{ cm}^{-1}$ .

FT-Raman spectroscopy was employed (i) to investigate the secondary structure of lysozyme and DNase I samples in aqueous state; (ii) to detect protein denaturation after thermal

stress in the solid state. Accordingly, the original FT-Raman spectra of lyophilised (as received) and spray-dried protein samples were collected for (i) aqueous solutions of lysozyme in water (7.5 and 20%, w/v) and DNase I in 5 mM Hepes buffer, pH 7.5, containing 1 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{NaN}_3$  (6%, w/v, before and after heating to  $90^{\circ}\text{C}$  in the HSDSC); (ii) solid states of DNase I and LDH (before and after thermal treatment in the DSC). Samples were analysed using a Bruker IFS-66 FT-IR spectrometer with FRA-106 FT-Raman module (Bruker, Germany). Sample excitation used a near infrared  $\text{Nd}^{3+}$ :YAG laser emitting at  $1064\text{ nm}$ . Both solids and solutions were studied at  $25 \pm 1^{\circ}\text{C}$ . Solids were analysed in stainless steel cups and solutions were assessed in a  $1\text{ cm}^3$  quartz cuvette with a mirrored rear surface, to enhance the intensity of the FT-Raman signal by multiple reflection of the FT-Raman radiation. The laser power was approximately 180–490 mW, for lyophilised (as received) and spray-dried solid samples and 900 mW for solutions. Spectra were the averages of 200 scans for solids and 4000 for solutions at  $4\text{ cm}^{-1}$  resolution. For aqueous solutions, reference spectra were recorded under identical scan conditions with water or buffer in the cuvette. The spectra of water or buffer were subtracted from the corresponding protein spectra. The FT-Raman module is equipped with a liquid nitrogen cooled germanium detector with an extended spectral bandwidth, which covered the wavenumber range  $3500$ – $500\text{ cm}^{-1}$ . A HeNe laser internally calibrates the spectrometer to ensure wavelength accuracy and the instrument performance (e.g. laser alignment) was checked using a sulfur standard prior to analysis.

## 2.7. Enzymatic assay

For lysozyme, biological activities of thermally denatured lyophilised (as received) and spray-dried lysozyme were determined after cooling (in HSDSC) to verify whether the renaturation is due to the nativeness of the protein structure, i.e. to correlate the folding reversibility with biological activity. In this 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 biological reaction was initiated by addition of 0.5 ml of each enzyme solution to 5 ml of the bacterial suspension. The activity unit of lysozyme is defined as the amount of enzyme decreasing the absorption rate at  $450\text{ nm}$  at  $0.001\text{ min}^{-1}$  at  $25^{\circ}\text{C}$  and pH 6.6 (Gorin et al., 1971). Rates were monitored using a UV/vis spectrophotometer (Pu 8700, Philips, UK) at  $25^{\circ}\text{C}$ .

The biological activity of DNase I samples was determined by the method provided by the protein supplier, based on that described by Kunitz (1950) in which 15 mg of the DNA substrate was dissolved in about 200 ml of water by allowing it to stand at  $4^{\circ}\text{C}$  overnight. The solution was stirred for 30 min and 37.5 ml of 1 M sodium acetate, pH 5.0 and 37.5 ml of 0.05 M  $\text{MgCl}_2$  were added and made up to 375 ml with water. The enzyme solutions of lyophilised (as received) and spray-dried DNase I samples were prepared by dissolving 2 mg enzyme/ml of 0.15 M NaCl. Then the prepared solutions were stored on ice and immediately prior to assay diluted to  $200\text{ }\mu\text{g}/\text{ml}$  in 0.15 M NaCl. To 30 ml of substrate solution was added 1 ml of enzyme solution and the increase in absorbance at  $260\text{ nm}$  at  $25^{\circ}\text{C}$  was recorded for  $\sim 15\text{ min}$ , using a UV/vis spectrophotometer (Pu



8700, Philips, UK). A vial of bovine pancreatic DNase I containing 2000 Kunitz units was used to standardise the DNase I assay.

The activity was determined using the maximum linear rate. Enzymatic activity assays measured the biological activity of DNase I before and after spray drying and determined the activity of the protein after heating to different temperatures (before and after  $T_m$ ).

For LDH, the enzymatic assay measured the biological activity of the protein in the lyophilised (as received) and spray-dried forms before and after heating to different temperatures (before and after  $T_m$ ). This protein was assayed for activity at 25 °C using procedures provided by Biozyme Laboratories Ltd. The method was based on the conversion of pyruvate by LDH in presence of nicotinamide-adenine dinucleotide, reduced form (NADH) to lactate and nicotinamide-adenine dinucleotide (NAD<sup>+</sup>). The amount of enzyme causing the oxidation of 1  $\mu$ mol of NADH per minute at 25 °C and pH 7.4 is a unit definition. The oxidation of NADH to NAD<sup>+</sup> leads to a decrease in absorbance at 340 nm.

Fresh solutions of 0.023 M sodium pyruvate and 0.006 M NADH were prepared in 0.005 M potassium phosphate, pH 7.4. Enzyme solution was prepared by dissolving 5 mg LDH to 1 ml of 0.05 M potassium phosphate, pH 7.4, kept ice-cold and immediately prior to assay diluted with 0.05 M potassium phosphate, pH 7.4, containing 1% bovine serum albumin.

Absorbance was adjusted to zero at  $\lambda = 340$  nm for a reference solution of 2.8 ml of 0.05 M potassium phosphate, pH 7.4, 0.1 ml of 0.006 M NADH and 0.1 ml of 0.023 M sodium pyruvate following equilibration of this solution to 25 °C. After addition of 0.1 ml enzyme solution to 2.7 ml of 0.05 M potassium phosphate, pH 7.4, 0.1 ml of 0.006 M NADH and 0.1 ml of 0.023 M sodium pyruvate, the rate of decrease in absorbance was measured at 340 nm, using a UV/vis spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, UK).

All data were presented as mean of three determinations  $\pm$  standard deviation. The Student's *t*-test assessed significance.

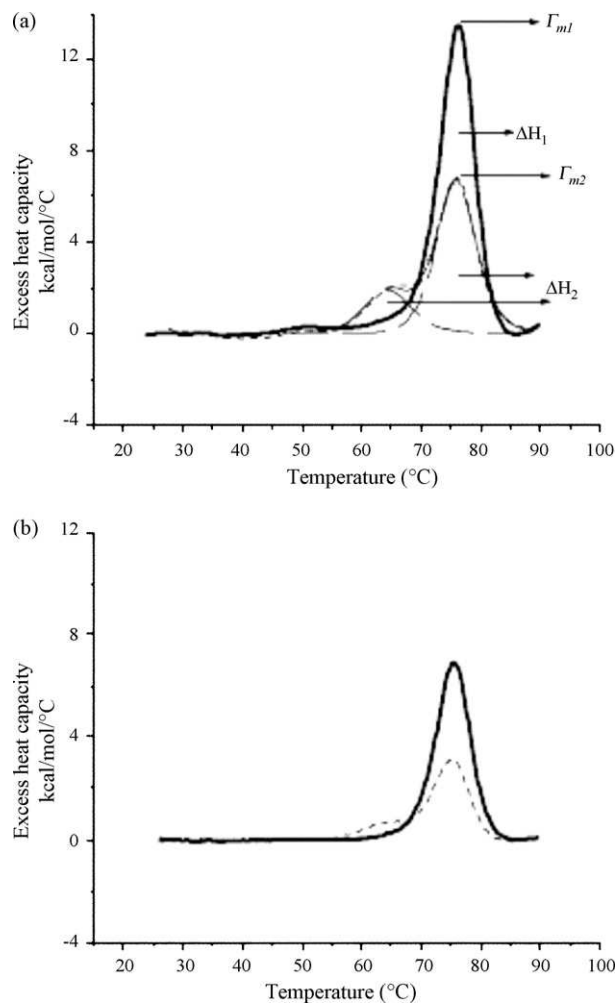
### 3. Results and discussion

#### 3.1. High sensitivity differential scanning calorimetry (HSDSC)

HSDSC monitored thermal stability and folding reversibility of reconstituted protein preparations. For lysozyme samples, traces for thermal denaturation and folding reversibility, using (UU) method, of lyophilised (as received) and spray-dried lysozyme are illustrated in Fig. 1a and b for 5 mg/ml and in Fig. 2a and b for 20 mg/ml protein concentrations.

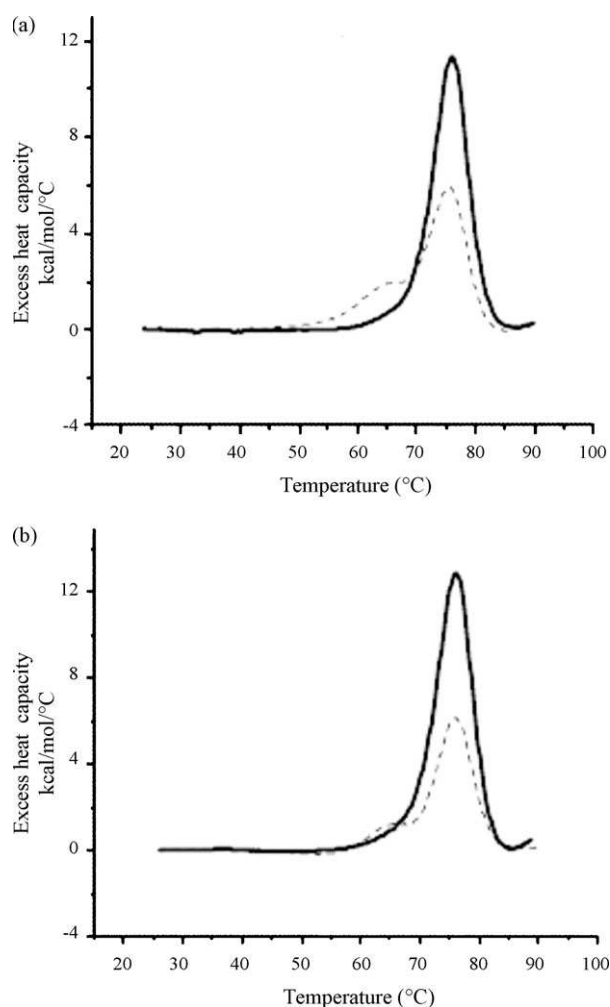
Thermodynamic parameters and enzymatic activities are in Table 2. As is evident in Figs. 1 and 2, HSDSC profiles of all samples showed a single endothermic peak (first upscan). Lysozyme started to unfold at  $\sim 65$  °C with a mean  $T_m$  of 76.1 °C ( $T_{m1}$ ).

It is noticeable that rescan profiles (endothermic, second heating cycle, Figs. 1 and 2 dotted lines) showed two peaks, a main one and a small peak or shoulder. Deconvolution of the data (using ORIGIN DSC data analysis software) revealed



**Fig. 1** – Normalised consecutive calorimetric upscans, first upscan (solid line) and second upscan (dotted line) with deconvolution (short dash) of lyophilised, as received (a) and spray-dried (b) lysozyme. Conditions: 5 mg/ml protein, 0.1 M sodium acetate buffer, pH 4.6, heating rate 1 °C/min.

two transition regions characterised by  $T_m$  at  $\sim 76.1$  °C ( $T_{m2}$ ) for the main peak and at  $\sim 66$  °C for the shoulder, indicating that the lysozyme transition is not a two-state transition. This may be explained on the basis that lysozyme consists of a single polypeptide chain divided into two structural domains ( $\alpha$ -helix and  $\beta$ -sheet) that are stabilised by different pathways (Buck et al., 1993). Otherwise, the rescan indicates that lysozyme was partially denatured thus indicating a lower enthalpy change ( $\Delta H$ ) for the main peak and a new transition resulting from the denatured protein. Additionally, Hirai et al. (1999) indicated that folding-and-unfolding kinetics of proteins depend on the number of amino acid residues. Proteins with residues above  $\sim 100$  do not obey simple two-state kinetics in a folding-and-unfolding process as a single cooperative unit. As lysozyme has 129 amino acid residues, it might follow more than two-state transitions; in other words, thermal transition of lysozyme may illustrate the formation of intermediates between native and denatured states.



**Fig. 2 – Normalised consecutive calorimetric upscans, first upscan (solid line) and second upscan (dotted line) of lyophilised, as received (a) and spray-dried (b) lysozyme. Conditions: 20 mg/ml protein, 0.1 M sodium acetate buffer, pH 4.6, heating rate 1 °C/min.**

For low protein concentration (5 mg/ml, Fig. 1a and b), Table 2 shows no significant difference between  $T_{m1}$  (transition temperature of first upscan) and  $T_{m2}$  (transition temperature of second upscan) for all samples. Additionally,

it is apparent that spray-dried lysozyme had  $T_m$ s similar to that of lyophilised (as received) protein, indicating that spray drying maintained the thermal stability of lysozyme. Table 2 reveals that the folding reversibility of spray-dried lysozyme was significantly less ( $p < 0.05$ ) than that of lyophilised (as received) material. Consequently, molecules from lyophilised protein were able to refold better than those from the spray-dried form.

With respect to samples with high protein concentration (20 mg/ml, Fig. 2a and b), Table 2 demonstrates that  $T_{m2}$  significantly decreased ( $p < 0.05$ ) compared to its corresponding  $T_{m1}$  for all samples. Also, spray-dried samples showed decreased  $T_{m1}$  compared with lyophilised (as received) material. Lyophilised protein revealed substantially increased ( $p < 0.05$ ) folding reversibility compared to the spray-dried form.

On comparing low and high protein concentrations, thermal stabilities ( $T_{m1}$  and  $T_{m2}$ ) for all samples at high concentration significantly decreased (probability for  $T_{m1}$  was  $< 0.05$  for lyophilised (as received) and  $< 0.001$  for spray-dried lysozyme and for  $T_{m2}$  was  $< 0.05$ ). Therefore, high protein concentration affects thermal stability, as  $T_m$  is protein concentration dependent. The change in  $T_m$  with protein concentration was ascribed to a coupling between folding and protein-protein interactions where the susceptibility of the monomer to denaturation relates to its association with other monomers (Branchu, 1999).

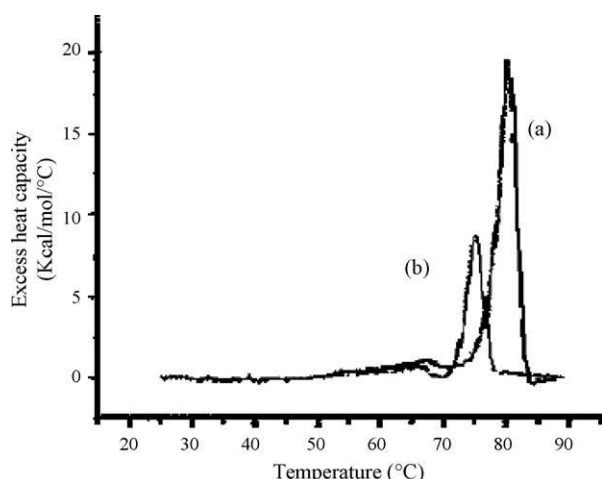
Furthermore, folding reversibilities and enthalpy changes of second upscans of all samples (Table 2) decreased with increasing concentration ( $p < 0.05$ ). Enthalpy change values correlated with the content of ordered secondary structure of protein (Koshiyama et al., 1981). The decrease in the enthalpy change of the protein may be due to denaturation at high protein concentration, since a partially unfolded protein requires less heat energy to denature completely than a native form. Also, high protein concentrations may promote protein-protein interactions that compete with the protein renaturation (Boye et al., 1997). Consequently, the renaturation of spray-dried lysozyme decreased ( $p < 0.05$ ) with increasing protein concentration, suggesting that the spray-dried protein is the form most affected by aggregation. The FT-Raman spectroscopy results confirm the HSDSC data (see later).

For DNase I samples, Fig. 3 shows the HSDSC thermal profiles of 10 mg/ml lyophilised (as received) and spray-dried DNase I in 0.15 M NaCl and 100 mM CaCl<sub>2</sub>, pH 6.3. Table 3 sum-

**Table 2 – Thermodynamic parameters for the thermal denaturation, folding reversibilities using consecutive upscan method (UU) and enzymatic activities of lyophilised (as received) and spray-dried lysozyme samples<sup>a</sup>**

Sample	$T_{m1}$ (°C)	$T_{m2}$ (°C)	% Folding reversibility ( $\Delta H_2/\Delta H_1$ )	% Enzymatic activity
Lyophilised				
5 mg/ml	76.1 ± 0.13	76.0 ± 0.15	59.5 ± 2.2	61.4 ± 2.0
20 mg/ml	75.9 ± 0.02	75.5 ± 0.04	51.6 ± 2.1	49.9 ± 3.2
Spray-dried				
5 mg/ml	76.1 ± 0.04	76.0 ± 0.14	50.6 ± 3.1	43.9 ± 1.1
20 mg/ml	75.5 ± 0.03	74.8 ± 0.06	42.5 ± 2.3	40.3 ± 2.3

<sup>a</sup>  $T_{m1}$  and  $T_{m2}$  are mid-point peak transition temperatures of first and second upscans;  $\Delta H_1$  and  $\Delta H_2$  are calorimetric enthalpies of transitions of first and second upscans; % enzymatic activity is the activity of each sample after thermal denaturation,  $\pm$ S.D.,  $n = 3$ . Accordingly, there was loss (ranging from 40 to 60%) in the biological activity of the protein due to the thermal effect.



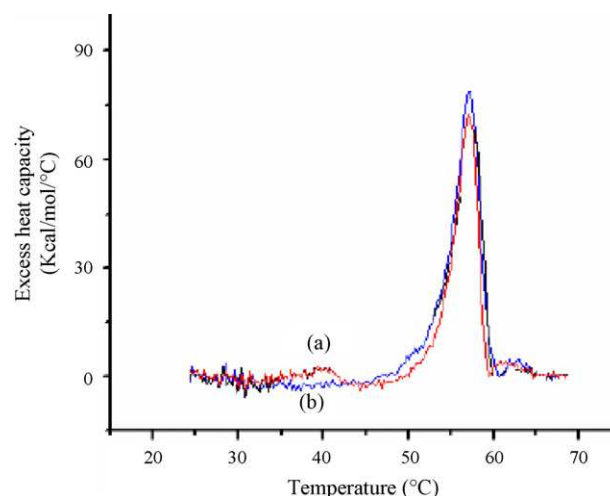
**Fig. 3** – Examples of normalised calorimetric data for the thermal denaturation of (a) lyophilised (as received) and (b) spray-dried deoxyribonuclease I. Conditions: 10 mg/ml DNase I, 0.15 M NaCl and 100 mM CaCl<sub>2</sub>, pH 6.3, heating rate 1.2 °C/min.

marises the apparent denaturation temperatures ( $T_m$ ) and enthalpy changes ( $\Delta H$ ) of the samples after fitting of each thermal transition peak to a single-peak non-two-state model. Fig. 3 and Table 3 indicate that spray-dried protein revealed decreased thermal stability, as its  $T_m$  was significantly lower than that of lyophilised (as received) DNase I ( $p < 0.05$ ). This may be explained by the presence of some denatured DNase I during spray drying. Thus, the  $T_m$  of the spray-dried form decreased compared with lyophilised (as received) material.

Upon rescanning, after cooling, of the DNase I samples, the endothermic peaks disappeared and aggregation was visible upon removal of the solution from the calorimeter. This means that DNase I samples were denatured.

Fig. 4 presents HSDSC plots for the thermal denaturation of lyophilised (as received) and spray-dried LDH. Table 3 summarises the thermodynamic parameters. Fig. 4 shows that the onset of LDH unfolding occurred between 48 and 50 °C. The mean  $T_m$  for lyophilised (as received) and spray-dried LDH samples was ~58 °C (Table 3).

The normalised data of spray-dried LDH (Fig. 4b) indicate single endothermic transition peak as for lyophilised (as received) LDH (Fig. 4a). LDH is a multimeric (tetramer) protein and thus can undergo more than one transition. There



**Fig. 4** – Examples of normalised calorimetric data for the thermal denaturation of (a) lyophilised (as received) and (b) spray-dried lactate dehydrogenase. Conditions: 5 mg/ml LDH, 0.05 M potassium phosphate buffer, pH 7.4, heating rate 1.2 °C/min.

may be intermediate formation during denaturation of the tetrameric LDH molecule. The detection and characterisation of these intermediates are complex (Creighton, 1994; Gaisford and Buckton, 2001). Upon rescanning, there was no reversibility of thermal transitions and LDH solutions gave precipitates. This implies complete denaturation of LDH. The exothermic peak at ~60 °C (Fig. 4) indicates aggregation. Single domain proteins (such as lysozyme, see above) more readily renature than complex proteins (such as DNase I and LDH). Therefore, special care should be taken during formulation of such large macromolecules to avoid their denaturation.

### 3.2. Differential scanning calorimetry (DSC)

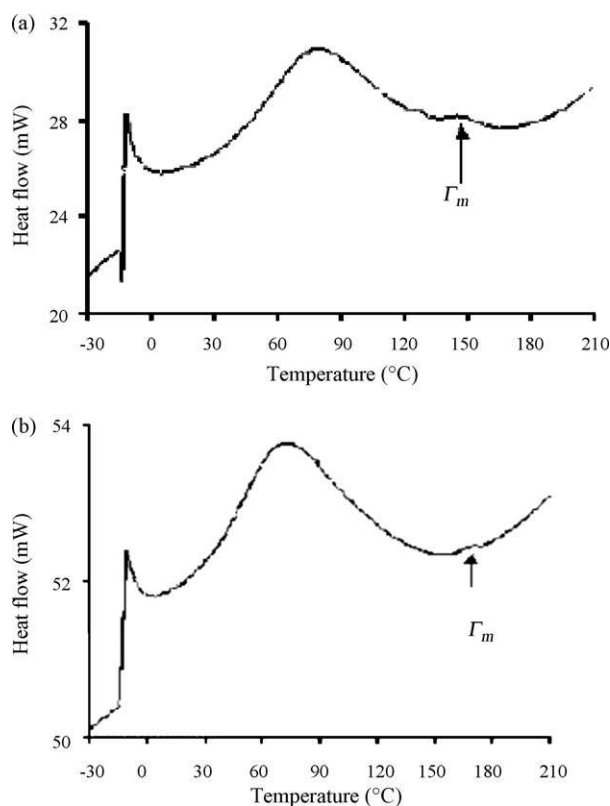
The thermal profiles of lyophilised (as received) and spray-dried DNase I samples are displayed in Fig. 5a and b. The profiles are characterised by broad endotherms at ~78 °C. This broad endotherm is characteristic for proteins and is due to the large internal heterogeneity of such molecules (Fan and Cooper, 1994) or dehydration (Chan and Gonda, 1998) or enthalpy relaxation in the solid (Pikal and Rigsbee, 1997). Also, DSC curves are characterised by another weak broad endothermic transition, representing the denaturation transition with some decomposition. The peak maximum reflects the apparent denaturation temperature ( $T_m$ ) as previously suggested for lysozyme (Elkordy et al., 2002). Enzyme powders are generally more stable in dry state than in solution with  $T_m$ s as high as 130 °C. The  $T_m$  values of lyophilised, as received (Fig. 5a), and spray-dried (Fig. 5b) DNase I were ~147.3 and 170.8 °C, respectively. The  $T_m$  of the spray-dried sample was higher than that of lyophilised (as received) material. This may be due to the difference in water content in these two forms, as dehydration of proteins by spray drying leads to removal of more water. This was confirmed by the thermogravimetric analysis; the water content of lyophilised DNase I as received ( $11.21 \pm 0.47\%$ , w/w) was significantly higher ( $p < 0.05$ ) than that of the spray-dried

**Table 3** – The apparent denaturation temperatures ( $T_m$ ) and calorimetric enthalpy changes ( $\Delta H$ ) of lyophilised (as received) and spray-dried deoxyribonuclease I (DNase I) and lactate dehydrogenase (LDH) in solution<sup>a</sup>

Process	$T_m$ (°C)	$\Delta H$ (kcal/mol)
Lyophilised DNase I	80.1 (0.15)	73.8 (5.9)
Spray-dried DNase I	74.8 (0.20)	32.3 (3.3)
Lyophilised LDH	58.1 (0.44)	62.7 (1.4)
Spray-dried LDH	58.3 (0.13)	77.2 (0.1)

<sup>a</sup> Values between brackets are S.D.,  $n = 3$ .



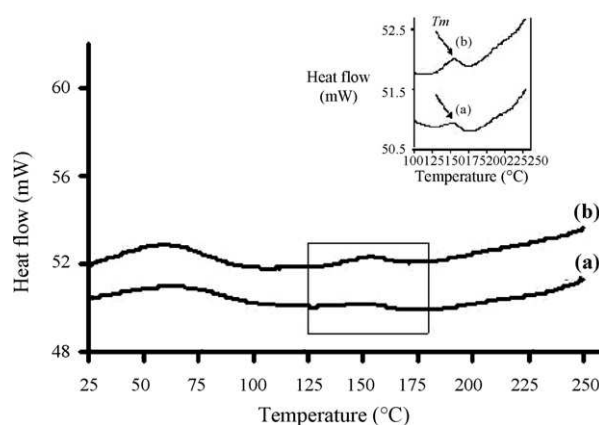


**Fig. 5 – Representative DSC thermograms of (a) lyophilised (as received) and (b) spray-dried deoxyribonuclease I (DNase 1). Conditions: heated from  $-30$  to  $210$  °C, heating rate  $15$  °C/min.**

protein ( $6.08 \pm 0.39\%$ , w/w). This may explain the higher stability of solid-state spray-dried DNase I over that of as received protein; as moisture increased, thermal stability decreased (Bell et al., 1995). Raised water content leads to increasing molecular mobility (Tzannis and Prestrelski, 1999) and hence decreased thermal stability or  $T_m$ .

Representative DSC traces of LDH samples are displayed in Fig. 6. The profiles of lyophilised, as received (Fig. 6a), and spray-dried (Fig. 6b) LDH are characterised by two thermal transitions. One is very broad at  $\sim 65$  °C and a second endotherm, which represents the apparent denaturation transition, at the peak maximum ( $T_m$ ) of  $152.8 \pm 0.4$  and  $153.5 \pm 0.2$  °C for lyophilised (as received) and spray-dried LDH, respectively. There was no significant difference ( $p < 0.05$ ) in  $T_m$  of lyophilised (as received) and spray-dried LDH. Thermogravimetric analysis of LDH samples showed that there was no significant difference ( $p > 0.05$ ) in weight losses of lyophilised and spray-dried LDH. The results were  $18.58 \pm 3.2$  and  $18.48 \pm 4.8\%$  (w/w) for lyophilised and spray-dried LDH, respectively.

Depending on the DSC data for DNase I and LDH, protein solid samples were heated to two temperatures one before the  $T_m$  ( $125$  °C for DNase I and  $150$  °C for LDH) and the other after the  $T_m$  ( $210$  °C for DNase I and  $250$  °C for LDH) to study the effect of heat to different extents on secondary structures of both proteins, using FT-Raman spectroscopy, and protein biological activities, via enzymatic assays. Before the  $T_m$ , the



**Fig. 6 – Representative DSC thermograms of (a) lyophilised (as received) and (b) spray-dried lactate dehydrogenase. Condition: heated from  $25$  to  $250$  °C at  $20$  °C/min. The insert indicates the expanded  $T_m$  region (marked in a box).**

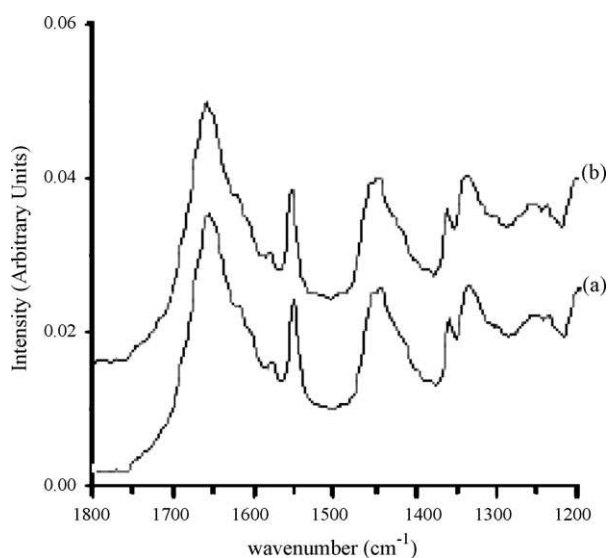
protein supposed to be in nature active state, whilst after the  $T_m$  the protein denaturation will take place.

### 3.3. Aqueous-state FT-Raman spectroscopy

#### 3.3.1. FT-Raman spectroscopy of lysozyme in aqueous state in low and high protein concentrations

FT-Raman spectroscopy characterised the conformational integrity of aqueous lyophilised and spray-dried lysozyme samples in low (7.5%, w/v) and high (20%, w/v) protein concentrations. Secondary structure was examined by comparison of FT-Raman spectra at regions: amide I (at  $\sim 1660$   $\text{cm}^{-1}$ ) for  $\alpha$ -helix and amide III ( $1250$ – $1350$   $\text{cm}^{-1}$ ) which are characteristic of proteins. The changes occur for these two bands in lysozyme related to a decrease of  $\alpha$ -helix with an increase of  $\beta$ -sheet and random coil structure (Jacob et al., 1998). The spectrum of lysozyme in aqueous solution (Fig. 7a, control spectrum, 7.5%, w/v) is similar to published data (Quinn et al., 1999). The plot shows the characteristic bands of lysozyme that exhibit  $\alpha$ -helical structure with amide I band at  $1660$   $\text{cm}^{-1}$ ; this band was mainly exploited to compare different spectra in water. Spectra of spray-dried samples prepared in low concentration (Fig. 7b) were identical to that of lyophilised (as received) lysozyme (control spectrum, Fig. 7a). These measurements were consistent with thermal stability from the HSDSC results, implying that spray drying maintained the native structure after reconstitution in low protein concentration.

For lyophilised (as received) and spray-dried samples prepared in high protein concentration (20%, w/v) lysozyme did not dissolve easily and FT-Raman spectra (Fig. 8) showed some perturbations compared to low protein concentration spectra (Fig. 7). Fig. 8a showed deterioration of lyophilised (as received) conformational integrity as there were shifts ( $-2$   $\text{cm}^{-1}$ ) in amide I band and ( $+5$   $\text{cm}^{-1}$ ) at  $1255$   $\text{cm}^{-1}$  and ( $-2$   $\text{cm}^{-1}$ ) at  $1335$   $\text{cm}^{-1}$ . Also, for spray-dried samples (Fig. 8b) there was perturbed amide I band and there was a shift in band at  $1335$   $\text{cm}^{-1}$  ( $+5$   $\text{cm}^{-1}$ ). Moreover, there was a new band at  $1750$   $\text{cm}^{-1}$ , attributed to aggregation (Souillac et al., 2002).

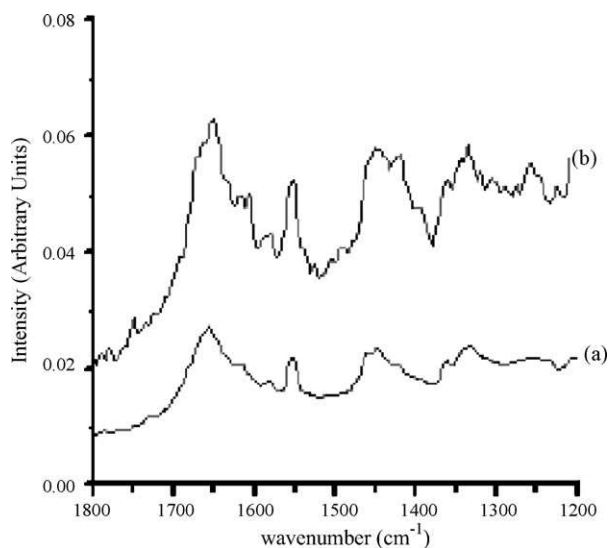


**Fig. 7** – Aqueous-state (7.5%, w/v) FT-Raman spectra over 1800–1200  $\text{cm}^{-1}$  of (a) lyophilised (as received, control) and (b) spray-dried lysozyme.

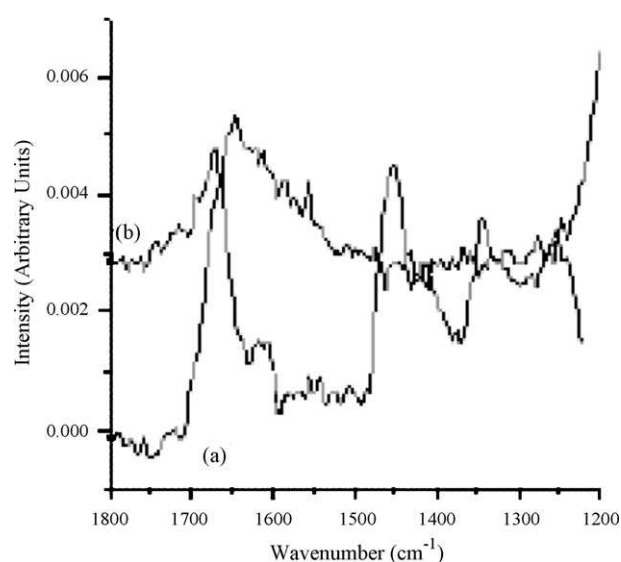
The perturbation in the secondary structure of all samples (Fig. 8) may be the cause of decreasing thermal stability of samples at high protein concentration (20 mg/ml) as indicated by HSDSC data.

### 3.3.2. FT-Raman spectroscopy of deoxyribonuclease I in aqueous state

Bussian and Sander (1989) assigned many vibrational modes of DNase I observed in Hepes buffer. FT-Raman spectra of lyophilised (as received) and spray-dried DNase I in 5 mM Hepes buffer, pH 7.5, containing 1 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{NaN}_3$  (6%, w/v) were identical (Fig. 9a—as received protein). The spectra agreed with Bussian and Sander (1989) although



**Fig. 8** – Aqueous-state (20%, w/v) FT-Raman spectra over 1800–1200  $\text{cm}^{-1}$  of (a) lyophilised (as received) and (b) spray-dried lysozyme.



**Fig. 9** – FT-Raman spectra of lyophilised (as received) deoxyribonuclease I (DNase I) in (a) 5 mM Hepes buffer, pH 7.5, containing 1 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{NaN}_3$  (6%, w/v, native solution) and (b) heated 5 mM Hepes buffer, pH 7.5, containing 1 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{NaN}_3$  (6%, w/v) to 90 °C (denatured solution) over 1800–1200  $\text{cm}^{-1}$ .

they reported a maximum intensity of the amide I band at 1662  $\text{cm}^{-1}$ ; but in our study the amide I band was at 1667  $\text{cm}^{-1}$ .

From the FT-Raman spectroscopy data, it is apparent that the spectra of processed DNase I samples were identical to the corresponding spectrum of lyophilised (as received) protein, demonstrating that spray-dried protein maintained the secondary structure of DNase I after reconstitution.

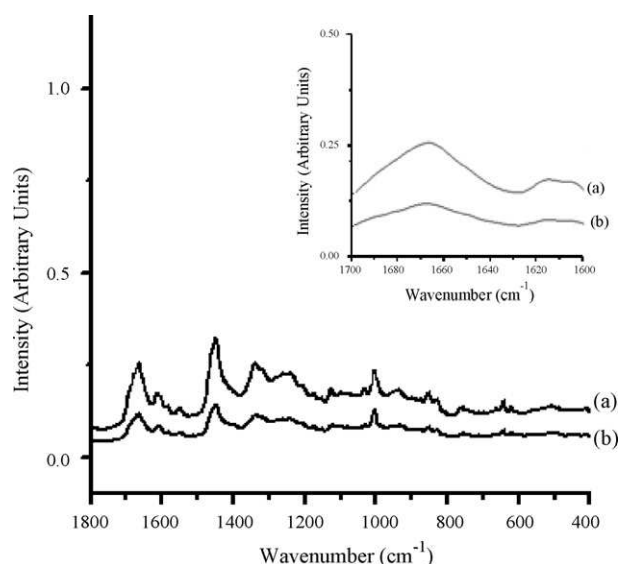
The spectrum (Fig. 9b) of lyophilised (as received) DNase I in 5 mM Hepes buffer, pH 7.5, after heating in HSDSC from 20 to 90 °C at 1.2 °C/min, changed compared with the control spectrum (Fig. 9a). The alterations included decreased intensity, broadening, splitting and shifting of amide I band; and increased signal:noise ratio and disappearance of some bands, indicating denaturation of the protein. The spectrum for heated spray-dried DNase I was similar to that of lyophilised (as received) protein. These results agree with HSDSC data (see above), as after heating of the samples in the microcalorimeter, aggregates were visible, implying protein denaturation.

### 3.4. Solid-state FT-Raman spectroscopy

FT-Raman spectroscopy was employed to study the conformational integrities of DNase I and LDH before and after heating of solid samples.

#### 3.4.1. FT-Raman spectroscopy of fresh (unheated) deoxyribonuclease I and lactate dehydrogenase solid samples

DNase I consists mainly of  $\beta$ -sheet, so amide I is at 1660–1680  $\text{cm}^{-1}$  and amide III is at 1220–1250  $\text{cm}^{-1}$  (Twardowski and Anzenbacher, 1994). Fig. 10 shows spectra of DNase I samples in the solid state over 1800–400  $\text{cm}^{-1}$ . The amide I band was at  $\sim$ 1667  $\text{cm}^{-1}$ , within the  $\beta$ -sheet range. Table 4 lists the peak



**Fig. 10** – FT-Raman spectra of (a) lyophilised (as received) and (b) spray-dried deoxyribonuclease I (DNase I) in the solid state over 1800–400  $\text{cm}^{-1}$ . The insert shows the amide I region (1700–1600  $\text{cm}^{-1}$ ).

positions and assignments of the vibrational modes for DNase I samples. The data for lyophilised (as received) DNase I agreed with that of Quinn's study (Quinn, 2000).

From Fig. 10 and Table 4, it is clear that spray-dried DNase I (Fig. 10b) showed some perturbation of conformational structure compared to the spectrum of lyophilised (as received) protein (Fig. 10a), as the intensities of amide I and III bands at

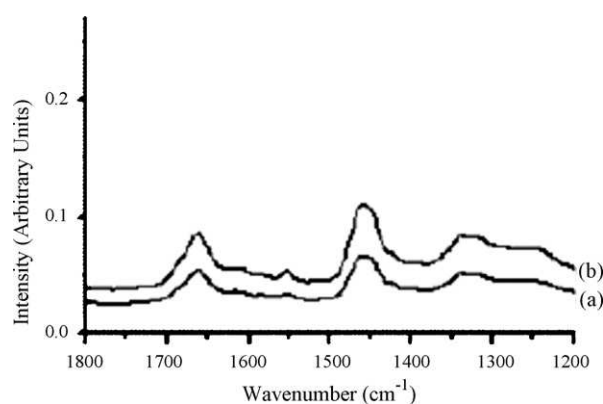
**Table 4** – FT-Raman peaks ( $\text{cm}^{-1}$ ), spectral assignments and vibrational main modes for lyophilised (as received) and spray-dried deoxyribonuclease I (DNase I) samples in the solid state<sup>a</sup>

Wavenumbers ( $\text{cm}^{-1}$ ) for DNase I		Assignments and vibrational modes <sup>b</sup>
Lyophilised	Spray-dried	
1667	1667	Amide I
1615	1613	Tyr, Trp, Phe ring
1554	1554	Trp
1449	1451	$\delta(\text{CH}_2)$
1341	1339	$\delta(\text{CH}_2)$ , Tyr
1242	1242	Amide III
1126	1127	$\nu(\text{C-N})$
1032	1041	Phe
1004	1003	Phe
854	854	Tyr
829	N.D. <sup>c</sup>	Tyr
759	759	Trp
644	644	Tyr
622	622	Phe
513	492	$\nu(\text{S-S})$

<sup>a</sup> Data are wavenumber position of bands obtained from Fig. 10.

<sup>b</sup>  $\nu$ : stretch;  $\delta$ : deformation; Tyr: tyrosine; Trp: tryptophane; Phe: phenylalanine.

<sup>c</sup> N.D., not detected.



**Fig. 11** – FT-Raman spectra of (a) lyophilised (as received) and (b) spray-dried lactate dehydrogenase in solid state over 1800–1200  $\text{cm}^{-1}$ .

1667 and 1242  $\text{cm}^{-1}$  decreased and a band at 1449  $\text{cm}^{-1}$  shifted to 1451  $\text{cm}^{-1}$ . Also, a band at 1341  $\text{cm}^{-1}$  moved to 1339  $\text{cm}^{-1}$ .

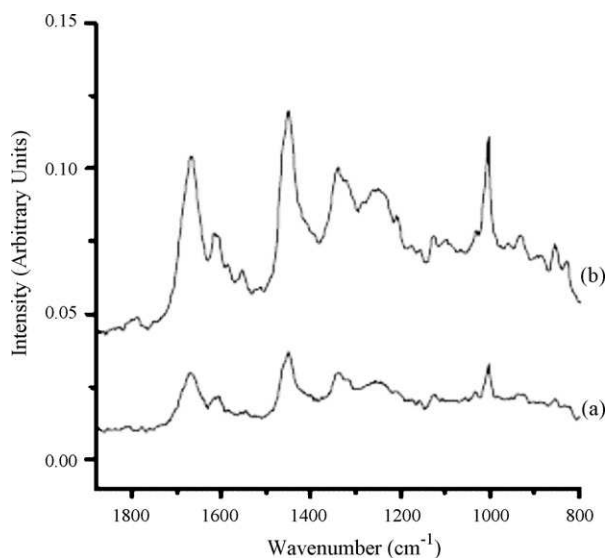
For LDH samples, LDH consists mainly of  $\alpha$ -helical structure, thus the amide I band in the FT-Raman spectrum is at 1645–1660  $\text{cm}^{-1}$ . Fig. 11 displays the spectra of lyophilised (as received) and spray-dried LDH solid samples over the range 1800–1200  $\text{cm}^{-1}$ . To the best of our knowledge, band assignments of vibrational modes of LDH FT-Raman spectra are not available in the literature. Accordingly, the spectra of spray-dried LDH samples (Fig. 11b) were compared with that of lyophilised (as received) LDH (Fig. 11a, a reference spectrum). Fig. 11a shows the amide I band at  $\sim$ 1660  $\text{cm}^{-1}$ , within the  $\alpha$ -helical range.

The spectrum of spray-dried LDH (Fig. 11b) showed the same position of amide I band at 1660  $\text{cm}^{-1}$  as lyophilised (as received) LDH (Fig. 11a), however the spray-dried form exhibited a small shift ( $\sim$ +3  $\text{cm}^{-1}$ ) in a band at 1337  $\text{cm}^{-1}$ .

#### 3.4.2. FT-Raman spectroscopy of denatured (thermally treated) deoxyribonuclease I and lactate dehydrogenase solid samples

Proteins can be heated to high temperatures, unfold and refold again. Unfolded protein can retain some of the native secondary structure (Manning et al., 1989). However, when proteins are heated to relatively high temperatures, they lose biological activity due to perturbations of the protein structure. Accordingly, lyophilised (as received) and spray-dried DNase I and LDH samples were heated in the DSC (see Section 2.4). Then, all samples were cooled immediately to 25  $^{\circ}\text{C}$  by the calorimeter-cooling unit and assayed for secondary structure, using FT-Raman spectroscopy, and biological activity. The practical significance for heating of proteins to such high temperatures is to study the effect of thermal stress on protein structure and performance. Consequently, overdrying of proteins at high temperature could be avoided (for example: during drying process) and stable protein products could be formulated.

Fig. 12 reveals the spectra of lyophilised (as received) DNase I after heating to 125  $^{\circ}\text{C}$ , before the  $T_m$  (Fig. 12a) and 210  $^{\circ}\text{C}$  (Fig. 12b) as an example of all heated samples. There were no differences in the spectra of heated lyophilised (as received)



**Fig. 12** – Solid-state FT-Raman spectra of lyophilised (as received) deoxyribonuclease I (DNase I) (a) heated to 125 °C and (b) heated to 210 °C over 1800–800  $\text{cm}^{-1}$ .

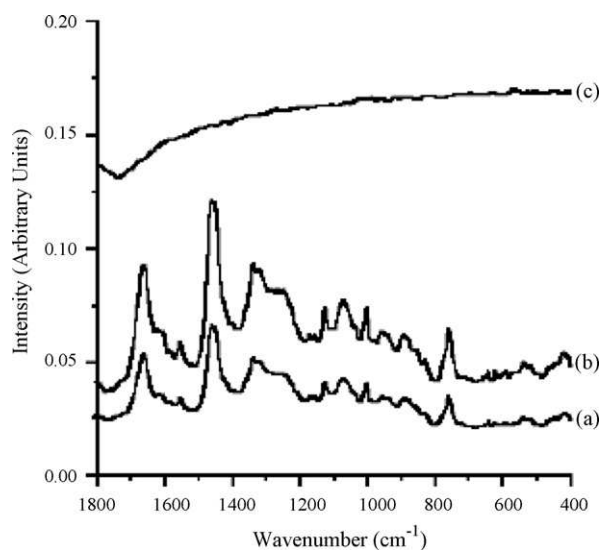
**Table 5** – FT-Raman spectral changes observed in deoxyribonuclease I samples (DNase I) after heating to 125 or 210 °C<sup>a</sup>

Wavenumber ( $\text{cm}^{-1}$ )	Changes on heating
1788	Appearance of a new band
1670	Broadening and shifting of amide I from 1667 $\text{cm}^{-1}$
1337	Shifting of a band at 1341 $\text{cm}^{-1}$
1250	Decreased intensity and shifting of amide III from 1242 $\text{cm}^{-1}$

<sup>a</sup> Data are wavenumber position of bands obtained from Fig. 12.

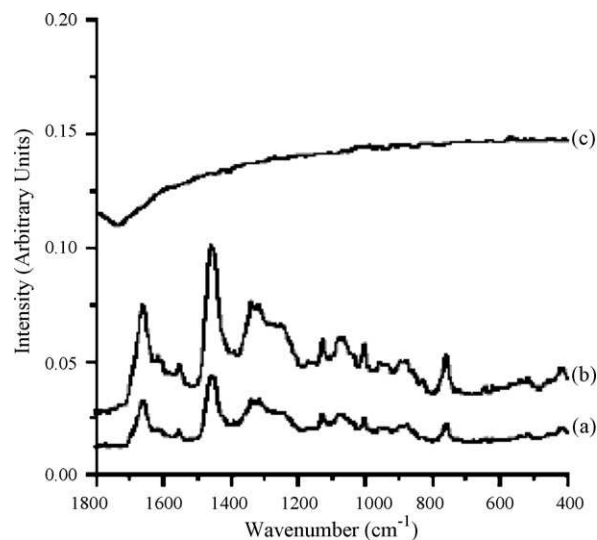
and spray-dried DNase I either to 125 or 210 °C, i.e. thermal stress affected all samples to the same extent. There was a new band appeared at 1788  $\text{cm}^{-1}$  in samples heated to 210 °C (Fig. 12b), indicating more thermal degradation on the protein at 210 °C. The band at this position (above 1700  $\text{cm}^{-1}$ ) indicates physical instability and aggregation (Souillac et al., 2002). Fig. 12 shows major changes with decreased intensities of all bands, compared with the control spectrum (Fig. 10a). The changes are summarised in Table 5. Fig. 12 and Table 5 indicate that thermal stress substantially perturbed the secondary structure of DNase I samples. Accordingly, heating of solid DNase I samples to temperatures below or above the apparent  $T_m$  of the solid protein degraded and hence denatured the protein (as indicated by substantial loss of activity, see below). The results for heated solid LDH samples (as will be illustrated later) are different from those of DNase I. LDH secondary structure altered marginally with retention of some activity upon heating to below its  $T_m$  but changed dramatically with complete loss of biological activity upon raising above its  $T_m$ . This indicates the diversity of proteins.

For LDH materials, Figs. 13 and 14 illustrate the FT-Raman spectra of thermally treated lyophilised (as received) and spray-dried LDH, respectively. For comparison, FT-Raman



**Fig. 13** – Solid-state FT-Raman spectra of lyophilised (as received) lactate dehydrogenase: (a) fresh, (b) heated to 150 °C and (c) heated to 250 °C over 1800–400  $\text{cm}^{-1}$ .

spectra of fresh solid lyophilised (as received) and spray-dried LDH are given in Figs. 13a and 14a, respectively, as controls. The spectra of LDH samples heated to 150 °C (before the  $T_m$ —Figs. 13b and 14b) exhibited small shifts in a band at 1554–1552  $\text{cm}^{-1}$  and in a band at 1337–1340  $\text{cm}^{-1}$  compared with fresh samples (Figs. 13a and 14a). Consequently, heated protein samples to a temperature before their  $T_m$  reveal minor alteration of the secondary structure. In contrast, heating of LDH samples to temperature above their  $T_m$  substantially change the conformation as indicated by disappearance of all characteristic bands of LDH after heating to 250 °C (Figures 13c and 14c). This indicates that LDH is sensitive to high temperature.



**Fig. 14** – Solid-state FT-Raman spectra of spray-dried lactate dehydrogenase: (a) fresh, (b) heated to 150 °C and (c) heated to 250 °C over 1800–400  $\text{cm}^{-1}$ .



From these data, it is apparent that heating of solid LDH samples to temperatures below their  $T_m$ s (determined by solid-state DSC) led to less detrimental effect on the protein structure than heating of LDH samples to temperature above their  $T_m$ s. Thermal stress affected the lyophilised (as received) and spray-dried LDH to the same extent because there were no variations in the FT-Raman spectra of heated lyophilised (as received) and spray-dried LDH either to 150 or 250 °C. FT-Raman spectroscopy is a useful method for detecting protein structural changes due to thermal stress. The structural changes caused by heat may lead to loss of biological activity of proteins. Hence, the effect of heat on activity was studied (see below).

### 3.5. Enzymatic assays

#### 3.5.1. Enzymatic assay for lysozyme samples

Lysozyme solutions upon cooling in the HSDSC after thermal denaturation were assayed for biological activity towards *M. lysodeikticus*. Based on the HSDSC results, all samples renatured to some extent after thermal stress. Thus, enzymatic assay should answer an important question. Is this renaturation or folding reversibility related to regain of the native structure of lysozyme (which is essential for biological activity), or does it result from misfolding, i.e. folding of the protein in a manner different from the original natured structure which subsequently leads to loss of activity?

Table 2 presents percentage enzymatic activities of preheated solutions, in HSDSC, of lyophilised (as received) and spray-dried lysozyme (relative to an aqueous solution of a fresh sample which represents 100% activity). It is evident that the biological activity of lyophilised (as received) lysozyme (5 mg/ml) was significantly higher than that of spray-dried form ( $p < 0.001$ ). Accordingly, lyophilised (as received) protein showed better recovery of enzymatic activity after thermal denaturation; this was consistent with the folding reversibility.

For samples containing high protein concentration (20 mg/ml), the results (Table 2) showed that the spray-dried material was less active compared with lyophilised (as received) ( $p \leq 0.001$ ), indicating perturbation of lysozyme structure as confirmed by FT-Raman spectroscopy data (see above). The enzymatic assay results answer the question posed previously in that folding reversibility was related to the native structure of lysozyme that is required for its activity, as the greater the folding reversibility, the higher the enzymatic activity.

From the HSDSC and enzymatic activity results, the folding reversibility, calculated by consecutive upscans, correlated with enzymatic activity of lysozyme.

#### 3.5.2. Enzymatic assays for DNase I and LDH samples

Enzymatic activity assays measured the biological activities of DNase I and LDH before and after spray drying and determined the activities of both proteins after heating to different temperatures (before and after  $T_m$ s). The recovered activities of spray-dried proteins were calculated as percentage relative to the aqueous solution of lyophilised (as received) proteins, i.e. the activity of lyophilised (as received) proteins was 100%. For fresh lyophilised (as received) and spray-dried DNase I

samples, the results were  $100 \pm 4.2$  and  $98 \pm 1.5\%$ . There was no significant difference for spray-dried samples compared with the lyophilised (as received) protein. This was consistent with FT-Raman spectroscopy data in aqueous solutions as spray-dried samples gave the same spectra as lyophilised (as received) protein after reconstitution. Also, the results indicated that spray-dried LDH retained 100% activity. This may be explained on the basis that spray-drying conditions allowed some residual moisture contents. This residual moisture content is essential for activity, i.e. water maintained the native structure at the active site cleft. This explanation is supported by Jiang and Nail (1998) who found that freeze-dried LDH had 100% recovered activity above a residual moisture level of about 20% and activity recovery decreased below this moisture level. They concluded that careful attention should be taken to avoid overdrying (during freeze- or spray-drying process) to maximise the recovery of activity from protein when no additives are present. In our study, the residual moisture content of spray-dried LDH, as determined by thermogravimetric analysis, was  $18.48 \pm 4.8\%$  (w/w) (see Section 3.2). Matzinos and Hall (1993) reported that at low outlet drying temperatures, during spray drying, LDH denaturation was low and the spray-dried powder was completely soluble, but at high outlet temperatures LDH denaturation was high and associated with powder insolubility.

For denatured (heated) DNase I samples, the residual biological activities after heating to 125 °C were 37% and the activities after heating to 210 °C were ~8% for all DNase I formulations. The results indicate that heating of DNase I samples to such temperatures denatured the protein and the extent of thermal degradation was increased with temperature.

FT-Raman spectra of heated lyophilised (as received) and spray-dried LDH materials (in DSC) indicated some conformational changes for samples heated below their  $T_m$  and gross changes for samples heated above their  $T_m$ . In this section, the effect of temperature on the tertiary structure was studied to correlate between structural changes observed in FT-Raman spectra of heated LDH samples and biological activity. The activity of unheated samples represents 100%. The results were 30%, for LDH samples heated to 150 °C (below the  $T_m$ ).

LDH samples heated to 250 °C (above the  $T_m$ ) revealed complete loss of activity, as the protein was completely insoluble. Hence, biological analysis of heated LDH samples supported their FT-Raman spectroscopy data.

From the above results, our suggestion for the primary source of instability of studied proteins due to heat effect is the instability of the protein tertiary structures. Our suggestion may be supported by the demonstration of Volkin and Middaugh (1992) who revealed that the native and active form of a protein are maintained by a weak balance of non-covalent forces (hydrogen bonds, ionic, hydrophobic and Van der Waals interactions). Upon heating, the heat disrupts these forces and protein molecules unfold; this unfolding leads to inactivation of the protein. Upon cooling, the non-covalent forces may re-form and the protein may be partially or fully activated because the combination of secondary and tertiary structures is thermodynamically favourable (Volkin and Middaugh, 1992). From point of view, the suggestion was confirmed by heating of proteins to high temperatures (above the  $T_m$ ) so proteins

lost most of their biological activity due to disruption of non-covalent forces of both tertiary and secondary structures. A research by Watanabe et al. (1993) showed that the stability of tertiary structures of mitochondrial tRNAs is dependent on stability of characteristic secondary structure. Moreover, a study by Miroliaei et al. (2007) suggests that sugars protected both secondary and tertiary structures of native and apo-yeast alcohol dehydrogenase from heat induced changes. This indicates a strong relationship between secondary and tertiary structure of proteins.

#### 4. Conclusions

HSDSC evaluated the conformational stabilities and folding reversibilities of lysozyme, DNase I and LDH in lyophilised (as received) and spray-dried forms. The overall results suggested that lyophilised lysozyme better refolded than spray-dried protein; the folding reversibility arises from the nativeness of the initial lysozyme structure as demonstrated by FT-Raman spectroscopy and biological activity data. Solid-state FT-Raman spectroscopy analysis indicated that dehydration of DNase I using spray drying perturbed somewhat DNase I conformation. For LDH samples, FT-Raman spectroscopy showed that spray drying of the protein did not greatly alter the secondary structure of LDH, supporting the results of HSDSC and biological assay. Consequently, spray drying of LDH is less detrimental compared to spray drying of lysozyme and DNase I. Moreover, the results indicated that enzymatic activity correlated with changes in the conformations of DNase I and LDH upon heating.

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