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Influence of protein on mannitol polymorphic form produced during co-spray drying

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ABSTRACT

The stabilizing ability of the excipient on pharmaceutically relevant proteins for potential therapeutic use is an extensive area of research but the effect the protein has on the excipient is rarely reported. The influence of two model proteins on the polymorphic behaviour of mannitol during spray drying was therefore investigated. Spray dried mannitol/protein blends were characterised structurally using X-ray powder diffraction (XRPD) and Fourier transform Raman spectroscopy (FT–Raman) and thermally by differential scanning calorimetry (DSC) and also thermogravimetric analysis (TGA). To assess the long term storage stability, samples were subjected to conditions of elevated temperature and relative humidity (RH). Structural and thermal analysis of the samples showed that upon spray drying mannitol could be completely amorphous or crystalline dependent on the protein co-spray dried. Upon storage at elevated temperature and RH different polymorphic forms of mannitol (beta and delta) were evident again dependent on the protein co-spray dried. Under the conditions employed there was a polymorph directing effect on mannitol dependent on the protein with which it was co-spray dried with co-solute effects on relative water levels being indicated as a major factor in directing the polymorph.

1. Introduction

Therapeutic proteins have inherent low physical stability of the native state. The stabilization of this structure is therefore essential if a protein is to be employed as a deliverable therapeutic agent. Due to the poor oral bioavailability of proteins, the parenteral route is a common method of delivery albeit at inconvenience to the patient of repeated injections which can lead to reduced compliance. Alternative delivery routes are attractive and the lungs provide this with their extensive absorptive surface. The formulation and delivery of therapeutic proteins for pulmonary delivery presents challenges due to the small particle sizes required and the high humidity of the respiratory tract. Spray drying is a suitable method of producing particles in a size suitable for inhalation. The spray drying of pharmaceuticals has been discussed at length by Broadhead et al. (1992). The process of spray drying has been used in the production of solid pharmaceuticals since the 1940s (Corrigan, 1995) and gives the formulator the opportunity to control parameters such as particle shape and size (Vanbever et al., 1999). The food industry also commonly employs spray drying to produce protein substances with enhanced stability (Sliwinski et al., 2004). Spray drying may be used to produce particles with a small size distribution and overall constituent uniformity (Nguyen et al., 2004). The spray drying of proteins with suitable stabilizing additives is a possible method of producing such particles for potential pulmonary delivery.

The application of spray drying to proteins requires avoidance of instability at elevated temperatures. The use of lower temperatures in the drying chamber may lead to inefficient drying and high residual water contents.

The spray drying of lysozyme has been investigated by Elkordy et al. (2002) who concluded that in the absence of added stabilizing agents the integrity of crystalline lysozyme exceeded that of spray dried lysozyme. Other sources of stress exerted during the spray drying process include air atomization rate which when increased can cause degradation (Maa et al., 1997). The effect of spray drying variables including feed flow rate, nozzle gas flow rate, inlet temperature and aspirator capacity on the production of insulin intended for inhalation have been summarised by Ståhl et al. (2002). Another principal consideration when selecting spray drying is the relatively low product yield which is typically 30–40% in a bench-top system (Maa et al., 1998). All of these factors have to be addressed when designing a spray dried protein formulation for potential pulmonary delivery.

Lactose is generally used as a carrier particle in dry powder inhalers (Taylor, 2002). The use of lactose as a possible carrier for therapeutic proteins in dry powder inhalers is not favoured as it has been shown to react with proteins upon formulation causing degra-

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dation (Andya et al., 1999). It is therefore necessary to investigate the use of alternative excipients for this purpose.

One such excipient, mannitol is used in food products and pharmaceutical formulations; in the latter it is used primarily as a diluent in tablet formulations (10–90%, w/w), and is also often used in chewable tablets. It is classified as non hygroscopic (Kibbe, 2000). The use of mannitol as a protein stabilizer in dried protein formulations has been widely reviewed (Bakaltcheva et al., 2007; Schule et al., 2007; Torrado and Torrado, 2002).

The intrinsic tendency for mannitol to recrystallize in freeze dried and spray dried protein formulations with low protein content can lead to phase separation (Costantino et al., 1998) and ultimately loss of therapeutic effect. The use of increased protein content or, more commonly, the addition of further co-solutes is often utilised to retard mannitol recrystallization in formulations (Izutsu et al., 2004, 2007; Pyne et al., 2003). Some co-solutes have limited use however as their long term adverse effects limits their potential use in pharmaceutical formulations (Hubbard, 1998).

The polymorphic form of mannitol present in a formulation can be influenced by the processing conditions employed in addition to the presence of co-solutes. For example, sample size (volume) has been shown to influence the polymorph of mannitol obtained in freeze dried systems (Telang et al., 2003) which was attributed to its crystallization in two stages. The mannitol:protein ratio has been shown to influence the stabilization obtained (Costantino et al., 1998). For example, it has been shown that for specific proteins that a 70% protein mass content is required to inhibit mannitol recrystallization, however this level of mannitol is not sufficient to stabilize against aggregation of the protein (Costantino et al., 1998). Sharma and Kalonia (2004) found that a level of 50% protein mass content was required to inhibit mannitol crystallization during vacuum drying with beta-lactoglobulin. From the above review which briefly discusses the factors influencing mannitol crystallization, much literature addresses the co-solute to mannitol ratios required to maintain mannitol in the amorphous state. It is not known why different proteins in the same weight ratio with mannitol could potentially influence the polymorphic behaviour of mannitol on drying. The present study probes protein:mannitol interaction using a standard spray drying procedure for two proteins. Lysozyme was chosen as the initial model protein because it has been reviewed and characterised extensively in the literature and readily available (Bonincontro et al., 1998; Careri et al., 1979; Makki and Durance, 1996; Malzert et al., 2002; Roxby and Tanford, 1971; Tomizawa et al., 1995). Trypsin was chosen as the second model protein because it differs in molecular weight and structure, yet has a similar pI to lysozyme. By standardizing spray drying conditions and protein:mannitol ratios before drying, the influence of the protein is emphasized.

2. Materials and methods

2.1. Materials

Chicken egg-white lysozyme (95% purity) and trypsin (peptidyl peptide hydrolase). Source: porcine pancreas (99.75% purity) were purchased from Biozyme Laboratories Limited, Gwent, UK. D-mannitol (α, β and δ polymorph) was obtained from Merck KGaA., Darmstadt, Germany. Proteins were stored desiccated at −20 °C and mannitol samples were stored in airtight, light resistant containers at room temperature until required.

2.2. Preparation of spray dried products

An aqueous solution of protein was dialysed (5%, w/v) using an 8 kDa membrane (Medicell International Ltd., Liverpool). To this solution was added the required amount of mannitol for each product to produce a 1:1 (w/w) protein/mannitol ratio. Each solution was spray dried using a Büchi 190 spray drier (Büchi, Switzerland). Samples were atomised using a two fluid nozzle (diameter 0.5 mm) into which the feed solution and air passed separately. The solutions were pumped to the nozzle through 1 mm silicone tubing at a flow rate of 12–13 ml/min and were dried with inlet temperatures of 135–145 °C to produce an outlet temperature of 60 ± 2 °C. Samples were passed through the nozzle with compressed air (6 bar pressure) and cooled by a continuous flow of cold water. The final product was separated from the air stream by a cyclone separator and the final product and collected manually from the receiver situated below the cyclone. Samples were then stored until analysis at 4 °C in a desiccator. Unprocessed samples of mannitol, trypsin and lysozyme were used as controls.

2.3. Structural analysis

Structural analysis incorporated X-ray powder diffraction (XRPD) and FT–Raman spectroscopy. All samples were analysed in triplicate.

2.3.1. X-ray powder diffraction (XRPD)

X-ray powder patterns were obtained using a Siemens D5000 Diffractometer (Siemens, Germany). The system comprised a monochromator with a Cu Kα radiation source (λ = 0.15418 nm) and a scintillation counter detector.

Samples were placed into a stainless steel holder and were subjected to manual compaction to obtain a level surface for analysis. The scanning range used was between 8° and 40° of 2θ with a stepwise scanning mode using a step size of 0.05° of 2θ and a step time of 3 s. Sample rotation of 30 rpm was employed during measurements.

2.3.2. Raman spectroscopy

Raman analysis was carried out using an FRA 106 Raman module with a Bruker IFS 66 optics system (Bruker, Germany). The Nd:Yag laser operated at 1.064 μm and the scattered radiation was detected by a liquid nitrogen-cooled, germanium detector which gave a spectral range of approximately 50–4000 cm^{-1}. Samples were manually compacted into an aluminium sample holder for analysis.

A laser power of 900 mW with 500 scans and a resolution of 4 cm^{-1} were used.

2.4. Thermal analysis

Thermal analysis incorporated differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). All samples were analysed in triplicate.

2.4.1. Differential scanning calorimetry (DSC)

DSC measurements were carried out using a PerkinElmer series 7 DSC thermal analysis system (PerkinElmer Ltd., UK). Samples weighing between 3 and 7 mg were crimped and sealed in aluminium DSC pans with vented lids which were placed in sample cells under nitrogen. Samples were scanned from 25 to 200 °C at 10 °C/min.

2.4.2. Thermogravimetric analysis (TGA)

TGA was employed to measure mass changes induced by thermal variation to determine the water content of the samples. Measurements were carried out in triplicate using a PerkinElmer TGA series 7 (PerkinElmer Ltd., UK). Solid samples between 1 and 8 mg were loaded onto an open platinum sample pan suspended from a microbalance and heated from 25 to 200 °C at 10 °C/min.

2.5. Accelerated stability study

Samples were placed into an oven at 40 °C in a sealed desiccator containing a saturated solution of sodium chloride to give a constant relative humidity of 75%.

Control samples were employed to establish the effects of spray drying on the samples. The controls used were physical mixtures of the raw starting materials used in equal proportions as in the spray dried samples.

3. Results and discussion

3.1. XRPD

Fig. 1 shows the XRPD patterns of the three polymorphic forms of mannitol.

The polymorphic forms of the reference powder patterns obtained were assigned using literature values for peak positions (Burger et al., 2000; Debord et al., 1987; Jones and Lee, 1970; Kim et al., 1986, Roberts et al., 2002). The three polymorphic forms exhibit unique peaks including; alpha (9.57° and 13.79°), beta (10.56, relatively intense peak at 14.71°) and delta (extremely intense peak at 9.74° then no peaks until 14.66°).

Fig. 2 shows the XRPD patterns of the spray dried protein/mannitol blends immediately after production. Because the overall aim of the work was to produce stable model protein formulations for potential pulmonary delivery the amount of mannitol as a co-solute ideally should be low enough to minimise the tendency of mannitol to recrystallize while retaining its thermal stabilizing ability during the spray drying process. We present data for the 1:1 (w/w) ratio of protein:mannitol similar to that identified by Costantino et al. (1998) and Sharma and Kalonia (2004). Interestingly in preliminary studies we evaluated three ratios 1:1, 1:9 and 9:1 (w/w) protein:mannitol ratios for both lysozyme and trypsin. Irrespective of the level of protein present, the X-ray data (not shown) indicated the same physical state of mannitol was obtained but that that state was different for each protein.

The results show that spray drying mannitol with trypsin produces an amorphous product which is evident as a characteristic amorphous halo in the XRPD pattern. The spray dried mannitol/lysozyme pattern indicates that some crystalline material is present within the sample but the peaks are reduced in intensity notably from the initial mannitol pattern shown in Fig. 1. This shows that the two proteins have different effects on the recrystallization pathway of mannitol with trypsin being more effective at inhibiting mannitol recrystallization. The XRPD patterns of the samples after 1 month storage at elevated temperature and RH are shown in Fig. 3.

The XRPD patterns show distinct differences in peak positions. As a result of reviewing the XRPD of the polymorphic forms shown in Fig. 1 it is possible to assign the polymorph present as delta in the mannitol/trypsin sample (characteristic intense peak at 9.74° of 2θ). It proved more difficult to assign the peaks in the mannitol/lysozyme sample as some are consistent with the delta polymorph and some are consistent with the beta polymorph as mentioned previously. It is however clear that they are not the same so further structural assignments may be made using Raman spectra.

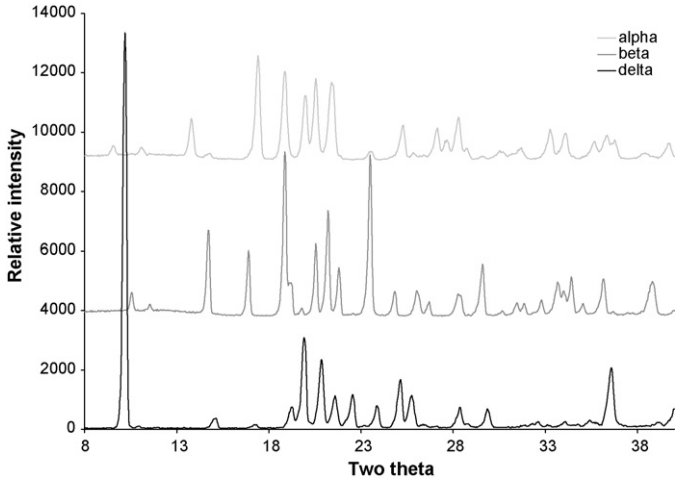

Fig. 1. XRPD patterns of the three mannitol polymorphic forms (commercially supplied).

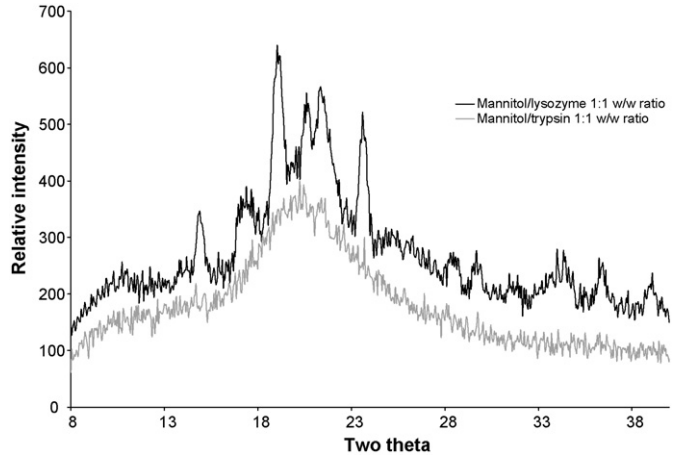

Fig. 2. XRPD patterns of spray dried mannitol/protein blends immediately after production.

3.2. FT–Raman

Fig. 4 shows the Raman patterns of the three mannitol polymorphic forms.

Fig. 4 highlights the hydroxyl (O–H) stretch region 2850–3050 cm^{-1} for the mannitol polymorphs. This region clearly indicates the differences between the polymorphic forms with each exhibiting unique peaks. These differences are due to the varying arrangements of the crystals within the lattice. This causes a difference in the hydrogen bonding networks of the samples and hence causes changes in the peak positions.

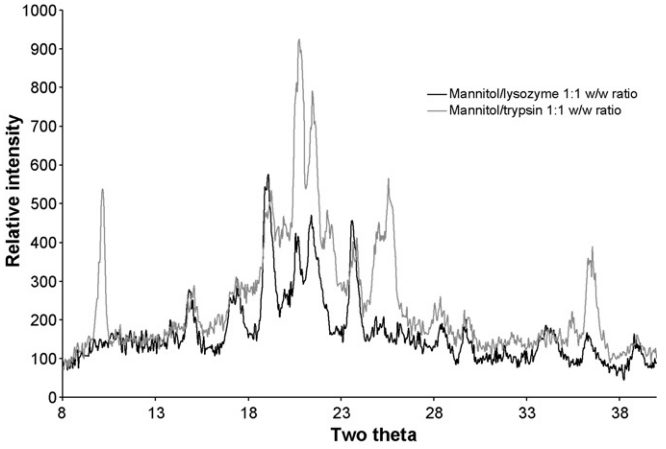

Fig. 3. XRPD patterns of spray dried mannitol/protein blends after 1 month storage at 75% RH and 40 °C.

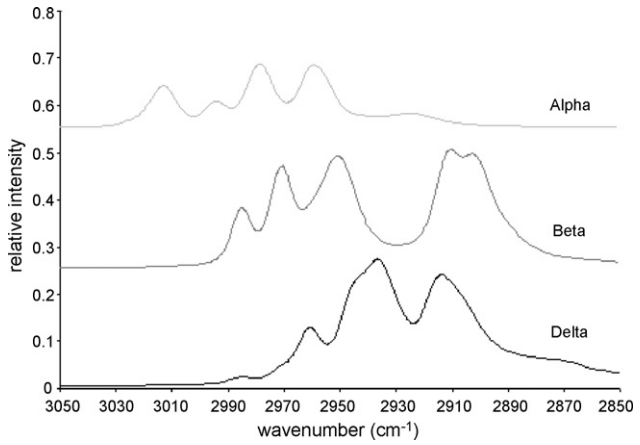

Fig. 4. Raman spectra of the three mannitol polymorphic forms (commercially supplied).

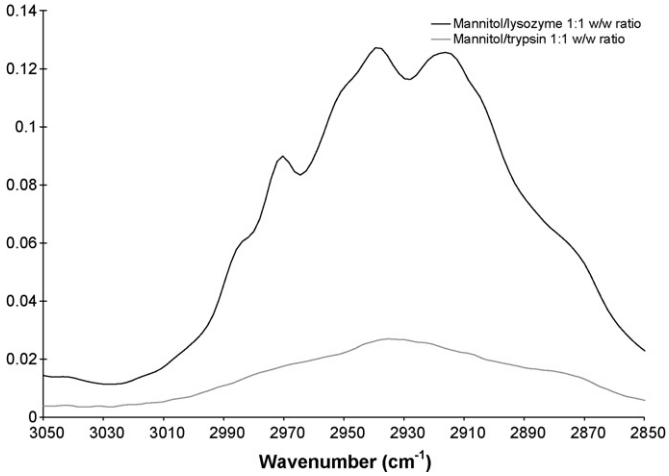

Fig. 5. FT–Raman spectra of spray dried mannitol/protein blends immediately after production.

Fig. 5 shows the Raman patterns of the spray dried protein/mannitol blends immediately after production.

The spectra are distinctly different in appearance with the mannitol/trypsin sample having no visible peaks. This is due to the amorphous character of the sample which is reduced on storage at elevated temperature and RH as indicated by XRPD. The Raman spectra of the samples after storage at elevated temperature and humidity are shown in Fig. 6.

As previously shown (XRPD) it is evident that the two proteins used in the experimental work do not contain the same mannitol polymorph after co-spray drying and storage. The XRPD data were difficult to assign for the samples and it was therefore necessary to determine polymorphic content using Raman spectra. From the reference spectra (Fig. 4) it was possible to assign the mannitol/lysozyme sample as containing the beta polymorph (peaks at 2900 and 2985 cm^{-1}) and the mannitol/trypsin sample as containing the delta polymorph (peaks at 2935 and 2960 cm^{-1}).

The Raman spectra of the mannitol/trypsin sample after storage at elevated temperature and humidity exhibited a peak in the 1500 cm^{-1} that was not evident immediately after production and was also not present in the mannitol/lysozyme samples.

This peak is consistent with the amino acid residue histidine (Brunner and Sussner, 1972; Lord and Yu, 1970; Tu, 1982). Histidine is catalytically essential in trypsin (Voet et al., 1999) and changes in this site could be reflected by a change in relative activity of the protein. The trypsin control sample exhibited a similar small histidine peak after exposure. It is possible that the spray drying of the polyols with trypsin stabilizes this native protein behaviour and this, therefore, may be an indication of stabilization of the native behaviour of the protein by these spray dried blends. This is consistent with previous findings (Hulse et al., 2008) where mannitol was shown to stabilize lysozyme during spray drying. It is however apparent that the peak is more distinct in the samples after 4 weeks exposure to 75% RH and 40 °C which may indicate that the effect is due to an increase in the moisture content of the sample. The imidazole ring present in the residue histidine is a tertiary amine and therefore highly nucleophilic and readily available to form hydrogen bonds. This makes the possibility of hydrogen bonding to water molecules a distinct possibility and could explain the increase in the peak at 1500 cm^{-1} which is characteristic of the N–H stretch region which would clearly be affected by such change in hydrogen bonding.

3.3. DSC and TGA thermal analysis

The DSC thermal profile of the mannitol/lysozyme spray dried blend both immediately after production and after 1 month storage at elevated temperature and humidity showed a single melt transi-

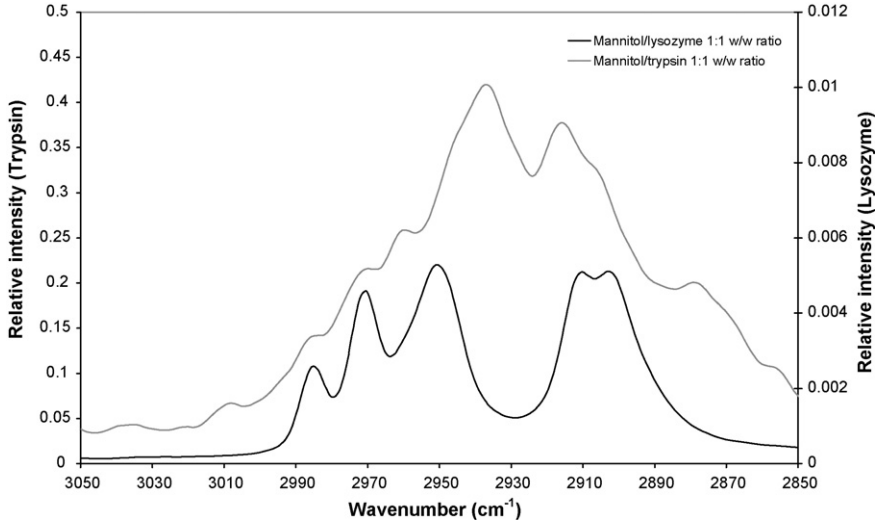

Fig. 6. FT–Raman spectra of spray dried mannitol/protein blends after 1 month storage at elevated temperature and RH.

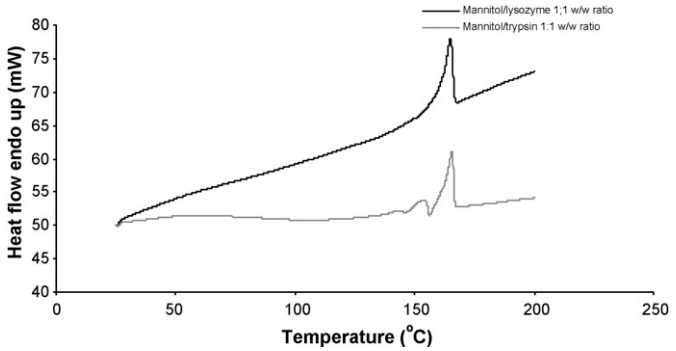

Fig. 7. DSC thermal profiles of spray dried mannitol/protein blends immediately after production.

tion of beta mannitol. The thermal profiles of the mannitol/trypsin spray dried blend both immediately after production and after storage at elevated temperature and humidity show two thermal transitions consistent with beta and delta mannitol as illustrated in Fig. 7.

The presence of delta mannitol in the thermal profiles of the trypsin containing samples is not evident in the XRPD or the Raman spectra as there is an interconversion of delta mannitol to the beta polymorph at elevated temperature (Burger et al., 2000). Thus DSC results were less reliable, compared to XRPD and Raman techniques, on providing insight into the polymorphic nature of mannitol in the samples.

The TGA results (Table 1) show that immediately after production there is no notable difference in water content between the two samples. This is however not the case after storage at elevated temperature and humidity. The blend containing lysozyme has a higher water content than the trypsin sample indicating that lysozyme preparation is more hygroscopic than trypsin. This was unexpected as the trypsin sample was amorphous immediately after production and would therefore be expected to have a higher water uptake. Mannitol is also known to form a hydrate but no X-ray evidence for its presence was determined in the present study. This finding points to the lysozyme preparation having relatively more moisture associated with its lysozyme component since it contains crystalline (non-hydrated) mannitol compared to the initial trypsin preparation containing amorphous mannitol. Following on from this, it would require lysozyme to have a greater affinity for water than trypsin. A structural explanation for this follows in the next paragraph.

Given the observation that the physical state of mannitol produced was independent of protein content over the ratio range 1:9–9:1 (w/w) but dependent on which protein was used indicates the properties of the protein to be important for the mechanism controlling which mannitol polymorph forms. The pI's of lysozyme and trypsin are 10.9 and 10 respectively suggesting their overall similar charge states would not be a major factor. However there are differences between the two proteins with lysozyme having a much greater percentage of ionised amino acid residues (Lys, Arg, His, Asp) than trypsin. The number of such residues is 20% in lysozyme compared to only 10% in trypsin. This suggests that whilst the neutral charge pH is similar for both proteins, the number of ionizable groups on lysozyme is greater, and may be expected to more strongly interact with water through ion–dipole interactions. Such a difference is consistent with the increased moisture content and hygroscopicity of the lysozyme sample.

Table 1
TGA results for spray dried samples ($n = 3$).

Sample	Mean water content (%) ± standard deviation
Spray dried mannitol/lysozyme immediately after production	2.99 ± 0.19
Spray dried mannitol/trypsin immediately after production	2.96 ± 0.46
Spray dried mannitol/lysozyme after One month storage at 40 °C and 75% RH	5.97 ± 1.45
Spray dried mannitol/trypsin after 1 month storage at 40 °C and 75% RH	3.16 ± 0.61

The residual water content may reflect the cause of the different polymorphic form of mannitol present in the spray dried protein/mannitol 1:1 (w/w) samples after exposure to 75% RH and 40 °C. Telang et al. (2003) showed that the delta form of mannitol was formed when smaller sample volumes were freeze dried compared to larger volumes which yielded the beta form. Yoshinari et al. (2002) also reports crystallization of the delta form from dilute aqueous acetone and used acetone washings to rapidly displace the water. From the above, the more rapid removal of water would favour the formation of the delta form. The beta form is stable with a lower solubility compared to the delta form (Roberts et al., 2002). The increased solubility of delta mannitol may be due to the tightly established hydrogen bond network of the delta polymorph reported by Yoshinari et al. (2003). It is possible that in the spray dried blends where trypsin is present, trypsin's postulated lower affinity for moisture and resultant lower water content facilitates the formation of the more metastable amorphous and delta forms, whereas the greater affinity of lysozyme for water retains more moisture to facilitate crystallization of the more thermodynamically stable delta form. Subsequently, the lower water content of the trypsin sample may inhibit the polymorphic delta–beta conversion, a transition which is known to occur when the moisture content of a sample is elevated (Yoshinari et al., 2002).

4. Conclusions

There are clear indications from this work that the two proteins interact differently when co-spray dried with mannitol. Whilst it remains unclear as to the exact mechanism causing this difference and whether it is one or a cumulative effect of several factors, the moisture content differences would implicate the relative affinity of the protein with water to be important in directing whether beta or delta mannitol crystallizes after spray drying. More studies on a wider range of proteins would lead to greater understanding as to how protein nature directs mannitol solid state form and help identify a more precise mechanism.

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