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Influences of copolymers (Copovidone, Eudragit® RL PO and Kollicoat® MAE 30 DP) on stability and bioactivity of spray-dried and freeze-dried lysozyme

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Abstract

Protein stability is the most crucial factor in protein pharmaceutical preparations. Various techniques were applied for producing stable protein formulations such as spray-drying and freeze-drying. However, heating and freezing stresses are disadvantages for proteins using these methods, respectively. Accordingly, excipients have been used to preserve therapeutic effects of proteins during processing and for long period of time. Therefore, influences of Copovidone, Eudragit® RL-PO and Kollicoat® MAE-30 DP (as excipients) on stability and integrity of lysozyme (as a model protein) in spray-dried and freeze-dried forms were investigated. Protein formulations in both dried forms were prepared without and with the addition of mentioned excipients at different concentrations. Protein formulations were characterised for yield determination, morphology using scanning electron microscopic (SEM), thermal analysis by Differential Scanning Calorimetry (DSC), secondary structure stability using Fourier transform infrared (FT-IR) spectroscopy and biological activity. All protein formulations were subjected to a stability study as solid protein formulations for 3 weeks at 24 °C/76% relative humidity and aqueous protein samples were stored at 50°C for 30 minutes in a water bath. Results showed that Copovidone successfully preserved integrity and biological activity of lysozyme before and after storage in both spray-dried and freeze-dried forms with more advantage for using higher concentration of the same excipient. Smooth spheres of spray-dried lysozyme formulations with Copovidone were smaller than spray-dried lysozyme without and with Kollicoat® MAE-30 DP, which affected %yield produced. Copovidone has demonstrated valuable protection ability for lysozyme.

Keywords: lysozyme, Copovidone, Eudragit® RL PO, Kollicoat® MAE 30 DP, stability, DSC, FT-IR, SEM, biological activity.
**Introduction**

Among all of the biological macromolecules, proteins embrace an exceptional heterogeneous class. Protein-based therapeutics has found to be an effective treatment for wide spectrum of diseases (1), e.g. diabetes, infections, inflammation, wound healing, decubital ulcers, sunburn etc. However, protein therapeutics suffers from the inadequate stability, especially in aqueous form (2), as a result of protein aggregation by the effect of protein unfolding or surface interaction between the hydrophobic residues within the proteins (3). This is consider the major drawback of such a drug. Proteins are marginally stable in solid form but prone for physical degradation.

Several methods were applied in order to overcome the challenges associated with protein stability. The most frequently used method to produce solid state protein formulations with a considerable stability is spray drying (see for example, (2,4,5) and freeze drying (see for example, (6-8). Another method used to stabilise proteins is by adding wide variety of excipients to stabilise proteins (see for example, (2,9,10).

Spray drying is a one-step liquid atomization technique wildly used to produce solid pharmaceutical dosage forms. This process can utilise micro- and nano-size scaled particles that are suitable for pulmonary administration (2). Protein spray-dried particles prepared using this technique were developed either alone or with the addition of some stabilising excipients. So far, different excipients were used in order to obtain a stable protein formulation using spray drying method include sugars (e.g. trehalose (10)), surfactants (e.g. pluronic F-127®(2), polyols (e.g. sorbitol (11)), polymers (e.g. dextran and polyethylene glycol (12)), antioxidants (e.g. ethylenediaminetetraacetic acid (13)), amino acids (e.g. ascorbic (13)), chelating agents (e.g. ammonium sulphate (14)).

Freeze drying method is a sublimation based technique commonly used for heat sensitive materials to increase their stability and shelf life as pharmaceutical products. This technique involves two steps: freezing and drying. A drastic reduction in the hydration of the proteins is a major denaturation factor in freeze drying process (15). Proteins are labile molecules that need to preserve their moisture content at certain level to ensure conformational structure and biological activity stability. However, in order to ensure long term stability of protein
pharmaceutical preparations, the moisture level shouldn’t exceed 9% which is enough to hydrate the active site cleft of the proteins (16). Some excipients has cryoprotective and lyoprotective properties accordingly used to stabilise proteins in freeze drying process (see for examples; hydroxyethyl cellulose used with lactate dehydrogenase (17), polysorbate 20, trehalose, β-cyclodextrin and hydroxylethyl starch with glucagon (18), trehalose with insulin (19), pluronic F68 with calcitonin (20) and maltotriitol, trehalose, maltitol, and lactitol with L-lactate dehydrogenase and bovine serum albumin (21).

The mechanism by which all of the additives works is not very clear. However, there are some suggested mechanisms: (i) excipients can replace the intermolecular interactions of water within the protein by the effect of dehydration, (ii) hydrating the active site cleft of the protein accordingly provide a good substrate (2,16) and (iii) direct interactions with the protein active site that assess reducing the potential energy of the protein by mutual exclusion of the hydrophobic residues of the protein exposed to the aqueous environment (2, 22) and (iv) vitrification (the formation of amorphous glass) that hinder any molecular motion and inhibit any kind of interactions between proteins which could lead to aggregation.

The purpose of this study is to investigate the effects of three copolymers named (Copovidone, Eudragit® RL PO and Kollicoat® MAE 30 DP) on spray-dried and freeze-dried lysozyme (as a model protein) thermal stability, integrity and biological activity before and after storage. Both drying processes were chosen for subjecting the protein to two different drying conditions using high temperature (during spray drying) and low temperature (during freeze drying). Copovidone is a copolymer of 1-vinyl-2-pyrrolidone and vinyl acetate (60:40 ratio). It is used in cosmetic and pharmaceutical preparations as a tablet binder, form a protective layer in film coating on tablets, film-forming agent in spray and effective in controlled drug release formulations. This excipient has a stabilising effect on lysozyme in aqueous media (23), however, its effect on lysozyme in dried forms is still unknown. Eudragit® RL PO is a copolymer of ethyl acrylate, methyl methacrylate and a few content of methacrylic acid ester with quaternary ammonium groups (as salts) that makes the polymer permeable (24). It’s usually used for sustained release drug delivery. Kollicoat® MAE 30 DP is a methacrylic acid-ethyl acrylate (1-1 mass proportion) copolymer used as a film-former in enteric coatings. Therefore, those polymers are worth investigation on protein stability. Kollicoat® MAE 30 DP and Eudragit® RL PO were also used as most of copolymers have
high potential to stabilise proteins. Lysozyme (a globular protein) was selected as a model protein as it is well characterised in the literature (for example 25, 26). Also, it was used due to its bacteriostatic and bactericidal activities, lysozyme is used in pharmaceutical industry (27) and food industry (28). Lysozyme was also found to have an inhibitory effect on HIV growth in vitro (29). The approach used in this study may be feasible to be applied to other proteins with lower thermal stability, to confirm this concept; trypsin has been used in this research to study its biological activity in the proposed formulations.

Materials and methods

Materials

Lysozyme was purchased from BBI Enzyme Ltd, UK. Copovidone, *Micrococcus Lysodeikticus* (lyophilised cells), sodium chloride and Sodium acetate anhydrous were obtained from Sigma-Aldrich, UK. Kollicoat® MAE 30 DP was purchased from BASF, Germany. Eudragit® RL PO was obtained from Rohm GmbH, Chemische Fabrik, Germany. Sodium hydroxide was purchased from BDH Chemical Ltd, UK. Potassium dihydrogen phosphate was purchased from Fisher Scientific, UK. Distilled water.

Preparation of spray-dried protein

Aqueous protein solutions (1%, w/v) were spray-dried without and with excipients (Copovidone or Kollicoat® MAE 30 DP) via a BÜCHI Mini Spray Dryer B-290. Excipients were used at different concentrations (the choice was based on some literature see for example Haj-Ahmad et al., 23) as follows: 0.2 and 0.5 % (w/v) of Copovidone and 2 and 4 % (v/v) of Kollicoat® MAE 30 DP dispersion (metha-acrylic acid: ethyl-acrylate copolymer 1:1 dispersion 30%). Solid proteins are known to be stabilised by excipients such as salts, sugars and polymers (30). Hence, the chosen copolymers were used in low concentrations ranging from 0.2% to 4% to study the sensitivity and response of dried lysozyme formulations to the stabilising effects, if there is any, of the small amounts of the used excipients. Protein solutions were filtered using 0.2 μm Cellulose Nitrate Membrane Filters (Whatman International Ltd.). The feed solution passed through a silicone tubing of inner diameter of 4 mm and peristaltic feed pump (35%) to an atomizing nozzle (0.7mm diameter) at rate of 7 ml/min and compressed air at rate of 600 l/h. Solutions were sprayed inside a glass chamber at an inlet temperature of 110± 4 °C and outlet temperature was 55 ± 3 °C. Cooling water was circulated through a jacket around the nozzle to minimise the heat stress effect on the
proteins. Spray-dried particles were collected by a high-performance cyclone separator and were stored tight in vials at 3-4 °C until further analysis.

**Preparation of freeze-dried protein**

Aqueous protein solutions (1%, w/v) were freeze-dried without and with (0.2 and 0.5 % (w/v)) Copovidone, (0.2 and 0.5 % (w/v)) Eudragit® RL PO and (2 and 4 % (v/v)) Kollicoat® MAE 30 DP. Freeze drying was performed using VirTis Benchtop Freeze Dryer Biopharma, USA. Two millilitres of protein formulations were filled into 5 mL glass vials. Solutions were let to freeze at -85 °C for 4 hr followed by lyophilisation for 48 hours at a pressure of 10mBar, condenser temperature of -100 ± 2 °C and shelf temperature of 21 °C. Shelf temperature was kept at 21 °C during the whole freeze drying process; meaning that protein samples were dried using primary drying step in which the sublimation of ice takes place and continuous vapour removal occurs due to the difference in vapour pressure of the samples compared to that of the condenser. The condenser temperature was set at low temperature (-100 °C) to allow for low residual moisture content (30). Hence, the secondary drying by increasing the shelf temperature above 21 °C has not taken place as it may lead to removal of essential bound water (by desorption) which may be crucial to proteins’ activity (16), nevertheless the effect of moisture on proteins is complex (30). The freeze-dried products were kept at 3-5 °C in desiccators containing silica gel until analysis.

**Characterisation of spray-dried and freeze-dried protein preparations**

**Determination of percentage yield**

The % yield was determined by defining the final weights of the prepared spray-dried protein particles. Then, % yield was calculated using the following equation:

\[
% \text{ Yield} = \frac{\text{Final protein weight}}{\text{initial protein weight}} \times 100
\]  
(Eq.1)

**Microscopic examination of spray-dried and freeze-dried lysozyme formulations**

The morphologies of the spray-dried and freeze-dried protein particles were inspected using scanning electron microscope (SEM) (Hitachi S3000-N variable pressure scanning electron microscope, Japan). Small quantity of the dried protein preparations were attached to a double-sided carbon tape (Agar Scientific, Stansted, UK), positioned on an aluminium stub. The samples were coated with a mixture of gold/palladium using a Quorum Technology
by exposing samples to an Argon atmosphere at about $10^{-1}$ mbar or 10 Pa. Samples were coated for $2 \times 10^5$ s.

**Structure analysis using Fourier Transform Infra-Red (FT-IR) Spectroscopy**

FT-IR spectroscopy was carried out using a Perkin–Elmer FT-IR Spectrum BX series (Beaconsfield, Buckinghamshire, UK) equipped with PIKE MIRacle™ detector. A small quantity of dried protein sample was loaded into the system. Peaks positions were detected using Spectrum BX series software version 2.19. The FT-IR spectra were recorded for protein samples and excipients, after subtraction of the background from 4000 to 550 cm$^{-1}$ at 4 cm$^{-1}$ resolution for an average of 25 scans.

**Thermal analysis of spray-dried and freeze-dried protein samples**

The thermal stability of lysozyme in all formulations was assessed in solid form by Differential Scanning Calorimetry (DSC). Freeze-dried and spray-dried protein samples were thermally analysed using DSC Q1000M TA instrument, England. Pure indium standard was used to calibrate the DSC instrument. Unprocessed, spray-dried and freeze-dried solid protein formulations (in the range of 2-4mg) were loaded into hermetically sealed pans. The pans were then loaded under nitrogen at a flow rate of 50ml/min. The pans were scanned from 0 °C to 300 °C at a rate of 10.0 °C/min. The thermographs were normalised in counter to lysozyme weight. All samples were analysed in triplicate.

**Biological activity assay for lysozyme**

The activity of lysozyme, in triplicate, was evaluated by monitoring the rate of hydrolysis of β-1,4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls by lysozyme (28). The bacterial suspension of *Micrococcus Lysodeikticus* (20%) was prepared in 90 ml phosphate buffer 0.067 M, pH 6.6, at 25 °C and 10ml of 1% sodium chloride (NaCl). Enzymatic solutions (15 μg/ml) of unprocessed, spray-dried and freeze-dried lysozyme without and with excipients were prepared using the same buffer. The biological reaction was initiated by adding 0.5 ml of each enzymatic solution to 2.5 ml of the bacterial suspension. The unit activity of lysozyme is well-defined as the total amount of lysozyme that decrease the absorption rate at of the system at $\lambda$ 450 nm by 0.001 min$^{-1}$ at
Activity (Units/mg) = \frac{\Delta 450\text{nm}/\text{min}}{0.001 \times \text{mg enzyme in the reaction}} \quad \text{(Eq. 2)}

Effect of heating (at 50°C) and relative humidity (75% RH) on lysozyme activity

Effect of stress conditions of high temperature and high RH on lysozyme formulations has been investigated. Accordingly, aqueous protein formulations were kept at 50°C for 30 minutes in a water bath. Solid protein samples were kept at accelerated conditions of 76% relative humidity (RH) at 24°C for three weeks. Samples were assessed post-storage for enzymatic activity (which considered the main effective test to investigate efficacy of the formulated enzyme) and the results were compared with the pre-storage samples.

Statistical analysis

The generated data were statistically analysed using SPSS®. Post Hoc test was used if data was normally distributed and Mann Whitney Test was used as non-parametric test if the data was not normally distributed. The P-value of less than 0.05 was considered as a significant level.

Result and discussion

Determination of percentage yields for spray-dried proteins

Spray drying was performed for lysozyme without and with Copovidone and Kollicoat® MAE 30 DP (Fig 1). No spray drying was performed for samples containing Eudragit® RL PO (Fig 1) due to insolubility of this excipient in liquid phase at room temperature (22°C) due to the presence of the salt quaternary ammonium groups in its structure (32).

Table 1 shows the percentage yields of spray-dried lysozyme formulations in absence and presence of excipients (Copovidone and Kollicoat® MAE 30 DP). All spray-dried formulations had more than 60% of yield although 30-40% of product yield is typically expected by using bench-top spraying system (33), however in pharmaceutical industry, the large scale production for spray dried pharmaceutical products is feasible and hence using a large scalable spray drier can lead to a highest possible yield. Spray drying of lysozyme with Copovidone (0.2 and 0.5% w/v) shows the lowest %yield and this is due to the small size of the spray-dried particles in this formulation as compared with spray-dried lysozyme and
spray-dried lysozyme with Kollicoat® MAE 30 DP. Copovidone has a relatively high glass-transition temperature (103-106 °C) that aids in fabricating small particle size. Spray drying system suffers from the inefficient particle collection of the small size particles that has a high impact on the %yield of the last product (2). Accordingly, particles with low density might be drawn up into the vacuum of the spray dryer (34). Fig 2 shows the big particle size of spray-dried lysozyme with Kollicoat® MAE 30 DP. This can also justify the highest (~70%) % yield in this formulation and the potential of the cyclone separator to capture the large size particles, therefore increasing the %yield. Kollicoat® MAE 30 DP is a copolymer which is designed for enteric tablet coating. During the spray drying process, this copolymer reduced the chance of the spray-dried particles to stick to the chamber walls and the cyclone separator of the spray dryer system. Thus, resulted in the highest spray-dried percentage yield for lysozyme samples.

Insert Fig 1 and Table 1

Microscopic examination of spray-dried and freeze-dried protein particles

The morphology of the protein solid formulations can be affected by the type of the used excipients and the applied processing technique. Fig 2 shows some selected SEM images of spray-dried and freeze-dried lysozyme formulations. Spray-dried protein particles were uniform, smooth and spherical architectures as compared with freeze-dried structures. Different types of additives were used, different effects on the morphology of the protein were observed.

Spray drying of lysozyme without excipients led to hollow spherical structures which remained the same when Kollicoat® MAE 30 DP was added. However, hollowness were disappeared when lysozyme was spray-dried with Copovidone. This shows that Copovidone has an effect on particle shape and density when spray-dried with the protein.

The morphology of spray-dried particles has a significant role in the aerodynamic properties and performance of aerosol applications (2). Prinn et al. (34) suggested four different morphologies of the spray-dried particles; (I) smooth spheres (such as some of the spray-dried lysozyme particles without excipients and most of spray-dried lysozyme particles with Copovidone) which are more preferable than other shapes as they can enhance the aerodynamic aerosol performance), (II) collapsed or dimpled particles (such as few particles
of the spray-dried lysozyme with no excipients and most of the particles of spray-dried
lysozyme with Kollicoat® MAE 30 DP) (III) particles with a ‘raisin like structure’ and (IV)
highly crumpled and folded particles (34).

Different factors have impacts on the morphology of spray-dried particles, particularly the
rate of drying, as faster drying would most likely to produce dimpled dried particles.
Subsequently, rapid evaporation of the liquid from the centre of the spherical particle/droplet
results in holes if the surface is solid and crusty, unless water can escape by diffusion (35). In
this study, the inclusion of Copovidone has improved the morphology of the spray-dried
protein particles. Copovidone might replace protein components at the droplet surface before
drying, accordingly, preserve the surface integrity of the spray-dried particles. Moreover,
Copovidone could diffuse the water out slowly and avoid protein denaturation by slowing
down the rapid dehydration of the protein. Copovidone’s ability to improve protein’s stability
was clearly demonstrated by the biological activity assay results.

Regarding freeze-dried protein formulations, the morphology of freeze-dried protein particles
is usually structured at the primary drying stage in the freeze drying process. Freeze-dried
lysozyme without excipients had relatively smooth surface, whereas freeze-dried protein in
combination with Eudragit® RL PO had very different, rough and very porous surface with
irregular morphology. Porous structure has higher surface area therefore may result in more
protein-oxygen contact which can provoke the oxidative degradation of the protein (36).
However, porous structure embraces a low density that can be an advantage for particles aim
for inhalation delivery if the particle size is controlled (37). When added as an excipient to
lysozyme, Copovidone resulted in the smoothest structure surface with no signs of crystals.
Accordingly, Copovidone have significantly reduced the crystallinity of lysozyme which was
also confirmed by DSC results. This indicates that lysozyme:Copovidone (1:0.2) formulation
produced amorphous structure. Eudragit® RL PO and Kollicoat® MAE 30 DP did not
produce smooth surface and had an adverse effect on the biological activity of lysozyme (as
will discuss later).

Insert Fig 2

Differential Scanning Calorimetry (DSC)

Thermal profiles of unprocessed, spray-dried and freeze-dried protein samples are shown in
Table 2 which represent heat flow as a function of temperature and show the apparent
denaturation temperature \((T_m)\) values of unprocessed and processed protein without and with excipients. All of the DSC thermogram scans are characterised by two or more endothermic peaks. One broad endothermic peak, around 100-130 °C, which is related to water content of lysozyme samples (28) and thus might give an indication about the water content within each formulation (10). The second endothermic peak with varying broadness, around 180-202 °C, the peak maximum was considered to represent the apparent denaturation temperature of the protein in the formulations \((T_m)\).

Insert Table 2

Drying processes (spray drying and freeze drying) of lysozyme led to a small reduction (by about 1 °C) of lysozyme apparent denaturation temperature as compared with the unprocessed lysozyme. A marked reduction of lysozyme’s apparent denaturation temperature was observed upon the addition of all excipients (Copovidon, Kollicoat® MAE 30 DP and Eudragit® RL PO). For spray-dried and freeze-dried lysozyme formulations with Copovidone, a significant \((P<0.05)\) reduction of \(T_m\) was observed upon increasing the Copovidone weight from 0.2 to 0.5% w/v (by ~10 °C). Moreover, there was an increase in the intensity of the endothermic water peak (first endothermic peak) in spray-dried samples of the lysozyme with Copovidone. This could be an indicative sign of the increase in water content in these formulations which might be due to the hygroscopic property of Copovidone which, in some instances, is considered as Copovidone’s limitation in its use that can affect the product stability in humid conditions. However, this can be overcome by a proper sealing and packaging of the final product. Spray drying and freeze drying of lysozyme with Kollicoat® MAE 30 DP showed a significant \((P<0.05)\) reduction of the apparent denaturation temperature of lysozyme with more reduction for samples with higher ratio of Kollicoat® MAE 30 DP (refer to Table 2). The thermal stability of lysozyme with Eudragit® RL PO was significantly \((P<0.05)\) higher than dried lysozyme samples with Copovidone and Kollicoat® MAE 30 DP. A third endothermic peak was observed for freeze-dried lysozyme with Kollicoat® MAE 30 DP or Eudragit® RL PO around 222-224 °C which might indicate decomposition of the formulations at this range of temperature.
The addition of the used excipients to the formulations led to broadness of the second endothermic peak as compared to unprocessed lysozyme (as received) which indicates a decrease in the crystallinity (38). This is further confirmed with surface morphological structures of the samples (absence of crystal structures) under SEM.

### Fourier Transform Infra-Red (FT-IR) spectroscopy

Infrared spectroscopy was used to determine the secondary structure of lysozyme and whether or not the used excipients (Copovidone, Kollicoat® MAE 30 DP and Eudragit® RL PO) managed to stabilise lysozyme conformational structure throughout the drying processes.

The secondary structure of proteins can be detected in the IR region of Amide I vibration (contributed to C=O stretching band with some contributions from CN stretching and CCN deformation) which can be detected in the range of 1600-1700 cm\(^{-1}\). Amide II vibration (contributed to the N-H bending vibration and C-N stretching) can be detected at the range of 1500-1600 cm\(^{-1}\) (39-40). FTIR spectroscopy analysis of lysozyme formulations was conducted within the range of 1800 – 900 cm\(^{-1}\). Fig 3 shows FT-IR spectra for unprocessed, spray-dried and freeze-dried lysozyme formulations.

**Insert Fig 3**

Unprocessed lysozyme (as received) had Amide I and II peaks at 1645 and 1538 cm\(^{-1}\), respectively. The biggest shift (+14 cm\(^{-1}\)) of Amide I peak was found for freeze-dried lysozyme: Eudragit® RL PO (1:0.5 weight ratio) sample (Fig 3j) as compared to the control lysozyme spectrum (Fig 3a). This was considered as the biggest change. Therefore, +/- 1 cm\(^{-1}\) was considered as minor shift and anything more than that was considered as major shift in peak position (28). Freeze drying of lysozyme without any excipient preserved the secondary structure and conformation integrity of lysozyme to a great extent in both Amide I and II bands (Fig 3c). Whereas, spray drying of lysozyme without excipients (Fig 3b) disturbed the secondary structure of lysozyme as there were major changes in the shapes and shifts in both Amide I and II bands. This was also confirmed by the biological activity results. A significant (p<0.05) reduction of the biological activity of lysozyme in the spray-dried sample (89.4±5.2%) was observed; while 99.4±3.9% activity of lysozyme was maintained by freeze drying of lysozyme sample with no excipients.

Freeze drying of lysozyme with Copovidone at two different concentrations (0.2 and 0.5% w/v) (Fig 3g,h, respectively), preserved the secondary structure and conformation integrity of
lysozyme. However, spray drying of lysozyme with Copovidone (0.2 and 0.5% w/v) revealed major shifts of Amide I band by +6cm⁻¹ (Fig 3d,e, respectively). From the above, it can be concluded that spray drying as a process for protein drying and without any excipients led to perturbation of the protein secondary structure.

Freeze-dried samples of lysozyme:Eudragit® RL PO (1:0.5) and lysozyme:Kollicoat® MAE 30 DP (1:4) showed major disruption of lysozyme secondary structure which is due to major shifts in both Amide I and II bands and this was combined with a significant (p<0.05) reduction of lysozyme biological activity in these samples, see below for protein biological activity results. This means that the above excipients at the mentioned concentrations triggered some sort of conformational changes to the secondary structure of the protein, accordingly, reduced the protein activity. In contrast, by using a lower concentration of Kollicoat® MAE 30 DP (in 1:2 lysozyme: Kollicoat® MAE 30 DP sample), freeze-dried lysozyme:Kollicoat® MAE 30 DP (1:2) resulted in major shift only at amide I band, accordingly, exhibited a higher biological activity (66.5±4.4%) as compared to lysozyme: Kollicoat® MAE 30 DP (1:4) (57.8±1.7%). Some literatures (e.g. (Vidal & Mello, 41)) have only focussed on the fact that the shift of Amide I band has a high impact on the protein biological activity. However, the results in this study exhibit the relevance of taking amide II into account when considering the analysis of protein bioactivity.

**Biological activity of lysozyme formulations before and after storage**

The biological activity of proteins is the most important aspect that reflects the success of any protein pharmaceutical formulation. Enzymatic activity assay measures the bioactivity of proteins that underwent dehydration stress and if the used excipients managed to protect the stability and integrity of the protein. Fig 4 displays the biological activity results of the reconstituted (freshly prepared, stored for 3 weeks at 7% RH at 24°C (as solid form) and the heated aqueous samples to 50 °C for 30 min) lysozyme samples without and with excipients (Copovidone, Kollicoat® MAE 30 DP and Eudragit® RL PO). The biological activity of the reconstituted protein formulations was expressed as a percentage ± SD relevant to the unprocessed protein (the activity of unprocessed protein was 100%).

Copovidone polymer, at both spray drying and freeze drying process, better maintained the biological activity and integrity of lysozyme after drying as compared with Kollicoat® MAE 30 DP and Eudragit® RL PO. Spray drying and freeze drying of lysozyme with Copovidone maintained the lysozyme activity when was used at higher ratio (1:0.5) (101.6±2.2 and
Accordingly, the addition of Copovidone at 0.5 weight ratio had retained the bioactivity of lysozyme at 100% as compared to unprocessed lysozyme, and significantly (p<0.05) improved its bioactivity as compared to spray-dried lysozyme without excipients. However, spray drying and freeze drying of lysozyme with Kollicoat® MAE 30 DP and Eudragit® RL PO led to a significant (p<0.05) reduction of protein’s activity as compared to unprocessed protein. Copovidone (Fig1a) is a hygroscopic polymer which has a possible ability to stabilise proteins by forming hydrogen bonds with the oxygen molecules at the protein active site and stabilise these bonds through the carbonyl acetate groups and carbonyl pyrrolidinone groups in its structure.

Lysozyme is considered as a relatively stable protein (thermally stable up to 75 °C), therefore to confirm the stabilising effects of the excipients, a sensitive protein (trypsin) was used in both spray dried and freeze dried forms with the same excipients and using same ratios as lysozyme to investigate this matter. The results were as follow: spray drying and freeze drying of trypsin with either Kollicoat® MAE 30 DP and Eudragit® RL led to horrendous reduction of trypsin biological activity (<30%). However, spray drying and freeze drying with Copovidone in both concentrations (0.2 and 0.5% w/v) significantly (p<0.05) helped to maintain more than 80% of trypsin biological activity (spray-dried trypsin with 0.2% w/w of Copovidone (94%), spray-dried trypsin with 0.5% w/w of Copovidone (87%), freeze-dried trypsin with 0.2% w/w of Copovidone (83%) and freeze-dried trypsin with 0.5% w/w of Copovidone (81%)). Accordingly, the effect of the used excipients was the same for both proteins (lysozyme and trypsin, which is more thermolabile compared to lysozyme).

Lysozyme formulations were subjected to stability study. It was found that unprocessed lysozyme had lost ~19.8% of its bioactivity when stored at high relative humidity and ~15% at high temperature, as compared with the unprocessed lysozyme before storage (Fig 4). More than 90% of lysozyme biological activity was preserved for freeze-dried and spray-dried lysozyme with Copovidone (using both weight ratios 1:0.2 and 1:0.5) compared to that of fresh protein formulations. Interestingly, the biological activity of protein was increased for freeze-dried lysozyme with Eudragit® RL PO.

All samples showed a significant reduction of proteins activity upon storage at 50°C for 30min except for spray-dried and freeze-dried lysozyme with Copovidone, spray-dried and freeze-dried lysozyme with 4% Kollicoat® MAE 30 DP and freeze-dried lysozyme with Eudragit® RL PO. This shows that these excipients help to rehydrate the protein and not just retain but...
improve its bioactivity during high temperature stress in contrary to protein samples without heating. DSC analysis showed similar results, Eudragit® RL PO and Kollicoat® MAE 30 DP have better thermal stability as they showed higher $T_m$ compared to Copovidone samples which could indicate why Eudragit® RL PO and Kollicoat® MAE 30 DP had lower bioactivity without being subjected to heat stress and improved bioactivity after subjecting to thermal (50°C for 30 minutes) stress. This suggest that some excipients can tolerate heat and absorb heat stress, not the protein included with those excipients.

A study by Dourado et al. (42) showed that Eudragit® L-100 which has a very similar chemical structure to the one used in this study (Kollicoat® MAE 30 DP) can form weak bound conjugates with proteins (38). Kollicoat® MAE 30 DP (Fig1b) contains several methyl groups in its molecular structure. It could possibly have been that Kollicoat® MAE 30 DP due to their several methyl groups in their molecular structure bound to lysozyme’s hydrophobic pockets on the enzyme surface, stabilizing it and at the same time blocking the active site. When lysozyme’s active site is blocked, it diminishes its bioactivity unless unblocked again. And only when heated at 50 °C for 30 minutes in aqueous solution, Eudragit® RL PO and Kollicoat® MAE 30 DP could have been hydrolysed off the enzyme releasing the enzyme and resulting in a higher bioactivity than the one before the heating.

Kollicoat® MAE 30DP works by its pH dependant solubility. It is used for enteric coating tablets and dissolves at pH above 5.5. It is advised by the manufacturer (BASF) to be protected from heat and frost. This is probably the reason why increased the concentration of Kollicoat® MAE 30DP in the protein samples reduced the retained bioactivity when freeze-dried. The pH of the phosphate buffer used in this study to dissolve the protein/Kollicoat® MAE 30DP mixture was higher than pH=5.5. It seems that Kollicoat® MAE 30DP has better high temperature tolerability than low temperature (during freeze drying).

**Conclusion**

Copovidone, a copolymer, significantly maintained the biological activity and conformation integrity of the protein as compared to Kollicoat® MAE 30DP and Eudragit® RL PO. Where spray drying and freeze drying of lysozyme with Copovidone preserved the lysozyme activity, when was used at the higher ratio (i.e. 1:0.5 protein:copolymer), at 100% as
compared to unprocessed lysozyme, and significantly (p<0.05) improved protein bioactivity as compared to spray-dried lysozyme without excipients.

Moreover, trypsin with Copovidone retained more than 80% of its biological activity after spray drying and freeze drying processes. Accordingly, the effect of the used excipients was the same for both proteins (lysozyme and trypsin). Therefore, it was concluded that Copovidone is a promising additive as it can preserve the integrity and activity of proteins using the two drying techniques. It is worth to be tried with more other proteins and with applying other formulating methods; such as protein crystallisation.

Declaration of Conflicts of Interest

Authors declare no conflict of interest

References


