Haj-Ahmad, Rita, Mamayusupov, M., Elkordy, E. A. and Elkordy, Amal (2016) Influences of copolymers (Copovidone, Eudragit RL PO and Kollicoat MAE 30 DP) on stability and bioactivity of spray-dried and freeze-dried lysozyme. Drug Development and Industrial Pharmacy, 42 (12). ISSN 0363-9045

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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.

1 **Introduction**

2 Among all of the biological macromolecules, proteins embrace an exceptional heterogeneous
3 class. Protein-based therapeutics has found to be an effective treatment for wide spectrum of
4 diseases (1), e.g. diabetes, infections, inflammation, wound healing, decubital ulcers, sunburn
5 etc. However, protein therapeutics suffers from the inadequate stability, especially in aqueous
6 form (2), as a result of protein aggregation by the effect of protein unfolding or surface
7 interaction between the hydrophobic residues within the proteins (3). This is consider the
8 major drawback of such a drug. Proteins are marginally stable in solid form but prone for
9 physical degradation.
10
11 Several methods were applied in order to overcome the challenges associated with protein
12 stability. The most frequently used method to produce solid state protein formulations with a
13 considerable stability is spray drying (see for example, (2,4,5) and freeze drying (see for
14 example, (6-8). Another method used to stabilise proteins is by adding wide variety of
15 excipients to stabilise proteins (see for example, (2,9,10).
16
17 Spray drying is a one-step liquid atomization technique wildly used to produce solid
18 pharmaceutical dosage forms. This process can utilise micro- and nano-size scaled particles
19 that are suitable for pulmonary administration (2). Protein spray-dried particles prepared
20 using this technique were developed either alone or with the addition of some stabilising
21 excipients. So far, different excipients were used in order to obtain a stable protein
22 formulation using spray drying method include sugars (e.g. trehalose (10)), surfactants (e.g.
23 pluronic F-127®(2), polyols (e.g. sorbitol (11)), polymers (e.g. dextran and polyethylene
24 glycol (12)), antioxidants (e.g. ethylenediaminetetraacetic acid (13)), amino acids (e.g.
25 ascorbic (13)), chelating agents (e.g. ammonium sulphate (14)).
26
27 Freeze drying method is a sublimation based technique commonly used for heat sensitive
28 materials to increase their stability and shelf life as pharmaceutical products. This technique
29 involves two steps: freezing and drying. A drastic reduction in the hydration of the proteins is
30 a major denaturation factor in freeze drying process (15). Proteins are labile molecules that
31 need to preserve their moisture content at certain level to ensure conformational structure and
32 biological activity stability. However, in order to ensure long term stability of protein

33 pharmaceutical preparations, the moisture level shouldn't exceed 9% which is enough to
34 hydrate the active site cleft of the proteins (16). Some excipients has cryoprotective and
35 lyoprotective properties accordingly used to stabilise proteins in freeze drying process (see
36 for examples; hydroxyethyl cellulose used with lactate dehydrogenase (17), polysorbate 20,
37 trehalose, β-cyclodextrin and hydroxylethyl starch with glucagon (18), trehalose with insulin
38 (19), pluronic F68 with calcitonin (20) and maltotriitol, trehalose, maltitol, and lactitol with
39 L-lactate dehydrogenase and bovine serum albumin (21).
40
41 The mechanism by which all of the additives works is not very clear. However, there are
42 some suggested mechanisms: (i) excipients can replace the intermolecular interactions of
43 water within the protein by the effect of dehydration, (ii) hydrating the active site cleft of the
44 protein accordingly provide a good substrate (2,16) and (iii) direct interactions with the
45 protein active site that assess reducing the potential energy of the protein by mutual exclusion
46 of the hydrophobic residues of the protein exposed to the aqueous environment (2, 22) and
47 (iv) vitrification (the formation of amorphous glass) that hinder any molecular motion and
48 inhibit any kind of interactions between proteins which could lead to aggregation.
49
50
51 The purpose of this study is to investigate the effects of three copolymers named
52 (Copovidone, Eudragit® RL PO and Kollicoat® MAE 30 DP) on spray-dried and freeze-
53 dried lysozyme (as a model protein) thermal stability, integrity and biological activity before
54 and after storage. Both drying processes were chosen for subjecting the protein to two
55 different drying conditions using high temperature (during spray drying) and low temperature
56 (during freeze drying). Copovidone is a copolymer of 1-vinyl-2-pyrrolidone and vinyl acetate
57 (60:40 ratio). It is used in cosmetic and pharmaceutical preparations as a tablet binder, form a
58 protective layer in film coating on tablets, film-forming agent in spray and effective in
59 controlled drug release formulations. This excipient has a stabilising effect on lysozyme in
60 aqueous media (23), however, its effect on lysozyme in dried forms is still unknown.
61 Eudragit® RL PO is a copolymer of ethyl acrylate, methyl methacrylate and a few content of
62 methacrylic acid ester with quaternary ammonium groups (as salts) that makes the polymer
63 permeable (24). It's usually used for sustained release drug delivery. Kollicoat® MAE 30 DP
64 is a methacrylic acid-ethyl acrylate (1-1 mass proportion) copolymer used as a film-former in
65 enteric coatings. Therefore, those polymers are worth investigation on protein stability.
66 Kollicoat® MAE 30 DP and Eudragit® RL PO were also used as most of copolymers have

67 high potential to stabilise proteins. Lysozyme (a globular protein) was selected as a model
68 protein as it is well characterised in the literature (for example 25, 26). Also, it was used due
69 to its bacteriostatic and bactericidal activities, lysozyme is used in pharmaceutical industry
70 (27) and food industry (28). Lysozyme was also found to have an inhibitory effect on HIV
71 growth in vitro (29). The approach used in this study may be feasible to be applied to other
72 proteins with lower thermal stability, to confirm this concept; trypsin has been used in this
73 research to study its biological activity in the proposed formulations.

74

75 Materials and methods

76 Materials

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

83 Preparation of spray-dried protein

84 Aqueous protein solutions (1%, w/v) were spray-dried without and with excipients
85 (Copovidone or Kollicoat® MAE 30 DP) via a BÜCHI Mini Spray Dryer B-290. Excipients
86 were used at different concentrations (the choice was based on some literature see for
87 example Haj-Ahmad et al., 23) as follows: 0.2 and 0.5 % (w/v) of Copovidone and 2 and 4 %
88 (v/v) of Kollicoat® MAE 30 DP dispersion (metha-acrylic acid: ethyl-acrylate copolymer 1:1
89 dispersion 30%). Solid proteins are known to be stabilised by excipients such as salts, sugars
90 and polymers (30). Hence, the chosen copolymers were used in low concentrations ranging
91 from 0.2% to 4% to study the sensitivity and response of dried lysozyme formulations to the
92 stabilising effects, if there is any, of the small amounts of the used excipients. Protein
93 solutions were filtered using 0.2 µm Cellulose Nitrate Membrane Filters (Whatman
94 International Ltd.). The feed solution passed through a silicone tubing of inner diameter of 4
95 mm and peristaltic feed pump (35%) to an atomizing nozzle (0.7mm diameter) at rate of
96 7 ml/min and compressed air at rate of 600 l/h. Solutions were sprayed inside a glass chamber
97 at an inlet temperature of 110± 4 °C and outlet temperature was 55 ± 3 °C. Cooling water was
98 circulated through a jacket around the nozzle to minimise the heat stress effect on the

99 proteins. Spray-dried particles were collected by a high-performance cyclone separator and
100 were stored tight in vials at 3-4 °C until further analysis.

101 **Preparation of freeze-dried protein**

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

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

118 **Determination of percentage yield**

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

121 % Yield= (Final protein weight)/(initial protein weight) *100 (Eq.1)

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

123 The morphologies of the spray-dried and freeze-dried protein particles were inspected using
124 scanning electron microscope (SEM) (Hitachi S3000-N variable pressure scanning electron
125 microscope, Japan). Small quantity of the dried protein preparations were attached to a
126 double-sided carbon tape (Agar Scientific, Stansted, UK), positioned on an aluminium stub.
127 The samples were coated with a mixture of gold/palladium using a Quorum Technology

128 (Polaron Range) SC760 by exposing samples to an Argon atmosphere at about 10^{-1} mbar or
129 10 Pa. Samples were coated for 2 × 105 s.

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

131 FT-IR spectroscopy was carried out using a Perkin–Elmer FT-IR Spectrum BX series
132 (Beaconsfield, Buckinghamshire, UK) equipped with PIKE MIRacle™ detector. A small
133 quantity of dried protein sample was loaded into the system. Peaks positions were detected
134 using Spectrum BX series software version 2.19. The FT-IR spectra were recorded for
135 protein samples and excipients, after subtraction of the background from 4000 to 550 cm^{-1} at
136 4 cm^{-1} resolution for an average of 25 scans.

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

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

146

147

148 **Biological activity assay for lysozyme**

149 The activity of lysozyme, in triplicate, was evaluated by monitoring the rate of hydrolysis of
150 β-1,4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid in
151 bacterial cell walls by lysozyme (28). The bacterial suspension of *Micrococcus Lysodeikticus*
152 (20%) was prepared in 90 ml phosphate buffer 0.067 M, pH 6.6, at 25 °C and 10ml of 1%
153 sodium chloride (NaCl). Enzymatic solutions (15 µg/ml) of unprocessed, spray-dried and
154 freeze-dried lysozyme without and with excipients were prepared using the same buffer. The
155 biological reaction was initiated by adding 0.5 ml of each enzymatic solution to 2.5 ml of the
156 bacterial suspension. The unit activity of lysozyme is well-defined as the total amount of
157 lysozyme that decrease the absorption rate at of the system at λ 450 nm by 0.001 min^{-1} at

158 24 °C. M501 Single beam Scanning UV/Visible spectrophotometer Camspec (Biochrom,
159 UK) was used to monitor lysozyme activity. The activity was calculated from the following
160 equation (31).

161 Activity(Units/mg)=Δ450nm/min/0.001×mg enzyme in the reaction (Eq.2)

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

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

169 **Statistical analysis**

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

174 **Result and discussion**

175 **Determination of percentage yields for spray-dried proteins**

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

180 Table 1 shows the percentage yields of spray-dried lysozyme formulations in absence and
181 presence of excipients (Copovidone and Kollicoat® MAE 30 DP). All spray-dried
182 formulations had more than 60% of yield although 30-40% of product yield is typically
183 expected by using bench-top spraying system (33), however in pharmaceutical industry, the
184 large scale production for spray dried pharmaceutical products is feasible and hence using a
185 large scalable spray drier can lead to a highest possible yield. Spray drying of lysozyme with
186 Copovidone (0.2 and 0.5% w/v) shows the lowest %yield and this is due to the small size of
187 the spray-dried particles in this formulation as compared with spray-dried lysozyme and

188 spray-dried lysozyme with Kollicoat® MAE 30 DP. Copovidone has a relatively high glass-
189 transition temperature (103-106 °C) that aids in fabricating small particle size. Spray drying
190 system suffers from the inefficient particle collection of the small size particles that has a
191 high impact on the %yield of the last product (2). Accordingly, particles with low density
192 might be drawn up into the vacuum of the spray dryer (34). Fig 2 shows the big particle size
193 of spray-dried lysozyme with Kollicoat® MAE 30 DP. This can also justify the highest (~
194 70%) % yield in this formulation and the potential of the cyclone separator to capture the
195 large size particles, therefore increasing the %yield. Kollicoat® MAE 30 DP is a copolymer
196 which is designed for enteric tablet coating. During the spray drying process, this copolymer
197 reduced the chance of the spray-dried particles to stick to the chamber walls and the cyclone
198 separator of the spray dryer system. Thus, resulted in the highest spray-dried percentage yield
199 for lysozyme samples.

200 **Insert Fig 1 and Table 1**

201

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

203

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

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

214 The morphology of spray-dried particles has a significant role in the aerodynamic properties
215 and performance of aerosol applications (2). Prinn et al. (34) suggested four different
216 morphologies of the spray-dried particles; (I) smooth spheres (such as some of the spray-
217 dried lysozyme particles without excipients and most of spray-dried lysozyme particles with
218 Copovidone) which are more preferable than other shapes as they can enhance the
219 aerodynamic aerosol performance), (II) collapsed or dimpled particles (such as few particles

220 of the spray-dried lysozyme with no excipients and most of the particles of spray-dried
221 lysozyme with Kollicoat® MAE 30 DP) (III) particles with a 'raisin like structure' and (IV)
222 highly crumpled and folded particles (34).

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

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

247 **Insert Fig 2**

248 **Differential Scanning Calorimetry (DSC)**

249 Thermal profiles of unprocessed, spray-dried and freeze-dried protein samples are shown in
250 Table 2 which represent heat flow as a function of temperature and show the apparent

251 denaturation temperature (T_m) values of unprocessed and processed protein without and with
252 excipients. All of the DSC thermogram scans are characterised by two or more endothermic
253 peaks. One broad endothermic peak, around 100-130 ^{o}C, which is related to water content of
254 lysozyme samples (28) and thus might give an indication about the water content within each
255 formulation (10). The second endothermic peak with varying broadness, around 180-202 ^{o}C,
256 the peak maximum was considered to represent the apparent denaturation temperature of the
257 protein in the formulations (T_m).
258
259
260
261 **Insert Table 2**
262
263 Drying processes (spray drying and freeze drying) of lysozyme led to a small reduction (by
264 about 1 ºC) of lysozyme apparent denaturation temperature as compared with the
265 unprocessed lysozyme. A marked reduction of lysozyme's apparent denaturation temperature
266 was observed upon the addition of all excipients (Copovidon, Kollicoat® MAE 30 DP and
267 Eudragit® RL PO). For spray-dried and freeze-dried lysozyme formulations with
268 Copovidone, a significant ($P<0.05$) reduction of T_m was observed upon increasing the
269 Copovidone weight from 0.2 to 0.5% w/v (by ~10 ºC). Moreover, there was an increase in the
270 intensity of the endothermic water peak (first endothermic peak) in spray-dried samples of
271 the lysozyme with Copovidone. This could be an indicative sign of the increase in water
272 content in these formulations which might be due to the hygroscopic property of Copovidone
273 which, in some instances, is considered as Copovidone's limitation in its use that can affect
274 the product stability in humid conditions. However, this can be overcome by a proper sealing
275 and packaging of the final product. Spray drying and freeze drying of lysozyme with
276 Kollicoat® MAE 30 DP showed a significant ($P<0.05$) reduction of the apparent denaturation
277 temperature of lysozyme with more reduction for samples with higher ratio of Kollicoat®
278 MAE 30 DP (refer to Table 2). The thermal stability of lysozyme with Eudragit® RL PO was
279 significantly ($P<0.05$) higher than dried lysozyme samples with Copovidone and Kollicoat®
280 MAE 30 DP. A third endothermic peak was observed for freeze-dried lysozyme with
281 Kollicoat® MAE 30 DP or Eudragit® RL PO around 222-224 ºC which might indicate
282 decomposition of the formulations at this range of temperature.
283

284 The addition of the used excipients to the formulations led to broadness of the second
285 endothermic peak as compared to unprocessed lysozyme (as received) which indicates a
286 decrease in the crystallinity (38). This is further confirmed with surface morphological
287 structures of the samples (absence of crystal structures) under SEM.

288 **Fourier Transform Infra-Red (FT-IR) spectroscopy**

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

292 The secondary structure of proteins can be detected in the IR region of Amide I vibration
293 (contributed to C=O stretching band with some contributions from CN stretching and CCN
294 deformation) which can be detected in the range of 1600-1700 cm^{-1}. Amide II vibration
295 (contributed to the N-H bending vibration and C-N stretching) can be detected at the range of
296 1500-1600 cm^{-1} (39-40). FTIR spectroscopy analysis of lysozyme formulations was
297 conducted within the range of 1800 – 900 cm^{-1}. Fig 3 shows FT-IR spectra for unprocessed,
298 spray-dried and freeze-dried lysozyme formulations.

299 **Insert Fig 3**

300 Unprocessed lysozyme (as received) had Amide I and II peaks at 1645 and 1538 cm^{-1},
301 respectively. The biggest shift (+14cm^{-1}) of Amide I peak was found for freeze-dried
302 lysozyme: Eudragit® RL PO (1:0.5 weight ratio) sample (Fig 3j) as compared to the control
303 lysozyme spectrum (Fig 3a). This was considered as the biggest change. Therefore, +/- 1cm^{-1}
304 was considered as minor shift and anything more than that was considered as major shift in
305 peak position (28). Freeze drying of lysozyme without any excipient preserved the secondary
306 structure and conformation integrity of lysozyme to a great extent in both Amide I and II
307 bands (Fig 3c). Whereas, spray drying of lysozyme without excipients (Fig 3b) disturbed the
308 secondary structure of lysozyme as there were major changes in the shapes and shifts in both
309 Amide I and II bands. This was also confirmed by the biological activity results. A significant
310 ($p<0.05$) reduction of the biological activity of lysozyme in the spray-dried sample
311 (89.4±5.2%) was observed; while 99.4±3.9% activity of lysozyme was maintained by freeze
312 drying of lysozyme sample with no excipients.

313 Freeze drying of lysozyme with Copovidone at two different concentrations (0.2 and 0.5%
314 w/v) (Fig 3g,h, respectively), preserved the secondary structure and conformation integrity of

315 lysozyme. However, spray drying of lysozyme with Copovidone (0.2 and 0.5% w/v) revealed
316 major shifts of Amide I band by +6cm^{-1} (Fig 3d,e, respectively). From the above, it can be
317 concluded that spray drying as a process for protein drying and without any excipients led to
318 perturbation of the protein secondary structure.

319 Freeze-dried samples of lysozyme:Eudragit® RL PO (1:0.5) and lysozyme:Kollicoat® MAE
320 30 DP (1:4) showed major disruption of lysozyme secondary structure which is due to major
321 shifts in both Amide I and II bands and this was combined with a significant ($p<0.05$)
322 reduction of lysozyme biological activity in these samples, see below for protein biological
323 activity results. This means that the above excipients at the mentioned concentrations
324 triggered some sort of conformational changes to the secondary structure of the protein,
325 accordingly, reduced the protein activity. In contrast, by using a lower concentration of
326 Kollicoat® MAE 30 DP (in 1:2 lysozyme: Kollicoat® MAE 30 DP sample), freeze-dried
327 lysozyme:Kollicoat® MAE 30 DP (1:2) resulted in major shift only at amide I band,
328 accordingly, exhibited a higher biological activity (66.5±4.4%) as compared to lysozyme:
329 Kollicoat® MAE 30 DP (1:4) (57.8±1.7%). Some literatures (e.g. (Vidal & Mello, 41)) have
330 only focussed on the fact that the shift of Amide I band has a high impact on the protein
331 biological activity. However, the results in this study exhibit the relevance of taking amide II
332 into account when considering the analysis of protein bioactivity.

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

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

343 Copovidone polymer, at both spray drying and freeze drying process, better maintained the
344 biological activity and integrity of lysozyme after drying as compared with Kollicoat® MAE
345 30 DP and Eudragit® RL PO. Spray drying and freeze drying of lysozyme with Copovidone
346 maintained the lysozyme activity when was used at higher ratio (1:0.5) (101.6±2.2 and

347 107.6±3.5%, respectively). Accordingly, the addition of Copovidone at 0.5 weight ratio had
348 retained the bioactivity of lysozyme at 100% as compared to unprocessed lysozyme, and
349 significantly ($p<0.05$) improved its bioactivity as compared to spray-dried lysozyme without
350 excipients. However, spray drying and freeze drying of lysozyme with Kollicoat® MAE 30
351 DP and Eudragit® RL PO led to a significant ($p<0.05$) reduction of protein's activity as
352 compared to unprocessed protein. Copovidone (Fig1a) is a hygroscopic polymer which has a
353 possible ability to stabilise proteins by forming hydrogen bonds with the oxygen molecules at
354 the protein active site and stabilise these bonds through the carbonyl acetate groups and
355 carbonyl pyrrolidinone groups in its structure.

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

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

375 All samples showed a significant reduction of proteins activity upon storage at 50C for 30min
376 except for spray-dried and freeze-dried lysozyme with Copovidone, spray-dried and freeze-
377 dried lysozyme with 4% Kollicoat® MAE 30 DP and freeze-dried lysozyme with Eudragit®
378 RL PO. This shows that these excipients help to rehydrate the protein and not just retain but

379 improve its bioactivity during high temperature stress in contrary to protein samples without
380 heating. DSC analysis showed similar results, Eudragit® RL PO and Kollicoat® MAE 30 DP
381 have better thermal stability as they showed higher T_m compared to Copovidone samples
382 which could indicate why Eudragit® RL PO and Kollicoat® MAE 30 DP had lower
383 bioactivity without being subjected to heat stress and improved bioactivity after subjecting to
384 thermal (50°C for 30 minutes) stress. This suggest that some excipients can tolerate heat and
385 absorb heat stress, not the protein included with those excipients.

386 A study by Dourado et al. (42) showed that Eudragit® L-100 which has a very similar
387 chemical structure to the one used in this study (Kollicoat® MAE 30 DP) can form weak
388 bound conjugates with proteins (38). Kollicoat® MAE 30 DP (Fig1b) contains several
389 methyl groups in its molecular structure. It could possibly have been that Kollicoat® MAE
390 30 DP due to their several methyl groups in their molecular structure bound to lysozyme's
391 hydrophobic pockets on the enzyme surface, stabilizing it and at the same time blocking the
392 active site. When lysozyme's active site is blocked, it diminishes its bioactivity unless
393 unblocked again. And only when heated at 50 ºC for 30 minutes in aqueous solution,
394 Eudragit® RL PO and Kollicoat® MAE 30 DP could have been hydrolysed off the enzyme
395 releasing the enzyme and resulting in a higher bioactivity than the one before the heating.

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

403

404 **Conclusion**

405 Copovidone, a copolymer, significantly maintained the biological activity and conformation
406 integrity of the protein as compared to Kollicoat® MAE 30DP and Eudragit® RL PO. Where
407 spray drying and freeze drying of lysozyme with Copovidone preserved the lysozyme
408 activity, when was used at the higher ratio (i.e. 1:0.5 protein:copolymer), at 100% as

409 compared to unprocessed lysozyme, and significantly ($p<0.05$) improved protein bioactivity
410 as compared to spray-dried lysozyme without excipients.

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

417

418 **Declaration of Conflicts of Interest**

419 Authors declare no conflict of interest

420

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