

46 **Abstract**

47 Bulk crystallisation of protein therapeutic molecules towards their controlled drug delivery is of
48 interest to the biopharmaceutical industry. The complexity of biotherapeutic molecules is likely to
49 lead to complex material properties of crystals in the solid state and to complex transitions. This
50 complexity is explored using batch crystallised lysozyme as a model. The effects of drying and
51 milling on the solid-state transformations of lysozyme crystals were monitored using differential
52 scanning calorimetry (DSC), X-ray powder diffraction (XRPD), FT-Raman, and enzymatic assay.
53 XRPD was used to characterise crystallinity and these data supported those of crystalline lysozyme
54 which gave a distinctive DSC thermogram. The apparent denaturation temperature (T_m) of the
55 amorphous lysozyme was ~ 201 °C, while the T_m of the crystalline form was ~ 187 °C. Raman
56 spectra supported a more α -helix rich structure of crystalline lysozyme. This structure is consistent
57 with reduced cooperative unit sizes compared to the amorphous lysozyme and is consistent with a
58 reduction in the T_m of the crystalline form. Evidence was obtained that milling also induced
59 denaturation in the solid-state, with the denatured lysozyme showing no thermal transition. The
60 denaturation of the crystalline lysozyme occurred mainly through its amorphous form.
61 Interestingly, the mechanical denaturation of lysozyme did not affect its biological activity on
62 dissolution. Lysozyme crystals on drying did not become amorphous, while milling-time played a
63 crucial role in the crystalline-amorphous-denatured transformations of lysozyme crystals. DSC is
64 shown to be a key tool to monitor quantitatively these transformations.

65 **KEYWORDS:**

66 Crystalline-amorphous-denatured transformations; Differential scanning calorimetry; FT-Raman;
67 Lysozyme crystals; Milling; X-ray powder diffraction.

68

69 **1. Introduction**

70

71 Lysozymes are a group of enzymes defined as 1,4- β -N-acetylmuramidases cleaving the
72 glycosidic bond in the bacterial peptidoglycan. Hen egg white lysozyme (HEWL) is a single chain
73 polypeptide of 129 amino acids cross-linked with four disulfide bridges resulting in a molecular
74 weight of 14307 Da [1]. HEWL has the ability to lyse bacteria, and therefore it has particular
75 interest for application in food and pharmaceutical products [2]. Previous researchers assured its
76 potent antimicrobial efficiency [3] and its safety [4]. Also, other research has resulted in improved
77 intranasal absorption and delivery [5] and lung delivery [6].

78 Zhou et al. [7] made lysozyme containing mats and they verified its excellent antibacterial
79 activity against *Escherichia coli* and *Staphylococcus aureus*, and therefore, these mats have
80 promising uses in antimicrobial packing, tissue engineering, and wound dressing. Sax and Winter
81 [8] prepared sustained release lysozyme containing implants using hot melt extrusion. Schlocker
82 et al. [9] used milling to prepare protein-loaded microparticles in industrial quantities. Milling has
83 also been used to prepare protein particles suitable for pulmonary delivery [10]. However, milling
84 and other pharmaceutical processes (e.g., drying, mixing) may produce uncontrollable variation of
85 protein solid states (i.e., crystal structure and crystal habit) and also loss of protein activity [11].
86 Different lyophilized solid forms of proteins have been shown to produce different dissolution
87 rates for reconstitution [12]. The stability of crystalline lysozyme has been shown to be greater
88 than that of the amorphous form [13,14]. Therefore, it is essential to monitor the solid state
89 transformations of lysozyme during pharmaceutical processes.

90 Differential scanning calorimetry (DSC) is a well-established and widely used technique
91 to monitor solid state transformations. However, the thermal transitions of lysozyme, in common

92 with other proteins, are usually characterized in the solution state, and often using a high-
93 sensitivity differential scanning calorimeter (HSDSC), which is capable of detecting the small
94 changes in enthalpy that arise when proteins unfold within their solutions [15]. Modulated
95 temperature differential scanning calorimetry [16] and thermally stimulated depolarized current
96 [17] have been tested as alternatives. However, the thermal transitions in the solution state cannot
97 differentiate the different solid forms. Therefore, researchers have studied the thermal transitions
98 of lysozyme in solid state using conventional solid-state DSC. However, their results did not
99 recognize the discrepancy between the thermal behaviours of the amorphous and crystalline
100 lysozyme powders [13,14,18,19].

101 There is a renewed interest in lysozyme, and its solid state form can have a significant
102 effect on dissolution and stability. We wished to explore how pre-treatment of lysozyme crystals
103 affected thermal behaviour, in an attempt to use thermal profiles as a fingerprinting indicator of
104 prior treatment. In this study, we prepare lysozyme crystals to be dried and/or milled, and
105 appropriate mixtures of the treated forms were prepared. Our aim is to use DSC to monitor the
106 potential solid state transformations of lysozyme during the treatment processes. We use Powder
107 X-ray diffraction (PXRD), FT-Raman, and enzymatic assay for reference. To our knowledge this
108 is the first application of DSC for the quantitative detection of crystalline, amorphous and
109 denatured lysozyme forms.

110

111 **2. Materials and methods**

112

113 *2.1. Materials*

114

115 Hen egg-white lysozyme (HEWL) (purity; 95%) (Biozyme Laboratories, UK),
116 *Micrococcus lysodeikticus* (Sigma-Aldrich), sodium chloride (NaCl) (99.5%) (Sigma-Aldrich) and
117 sodium acetate anhydrous (purity; 98%) (BDH Chemicals Ltd., Poole, UK) were purchased as
118 indicated. The purchased lysozyme sample was considered to be unprocessed lysozyme. Water
119 was deionised and double distilled.

120

121 *2.2. Sample Preparation*

122

123 *2.2.1. Preparation lysozyme crystals using batch crystallization method*

124

125 One litre of a solution of lysozyme 4 % w/v in sodium acetate buffer (pH 4.6; 0.1 M) and
126 one litre of a solution of sodium chloride 10 % w/v in the same buffer were separately passed
127 through a 0.2 micron filter and then mixed in a glass container. The produced solution contained
128 2% w/v lysozyme and 5% w/v NaCl. This solution was then sealed and kept for ten days at 20 °C.
129 Crystals formed were collected by filtration. Adsorbed water was removed by air drying (5 h).
130 These procedures were used to prepare lysozyme crystals by a batch crystallisation method [13].

131

132 *2.2.2. Preparation of dried lysozyme crystals*

133

134 A glass column of dimensions 2m (three loops) × 6mm (outer diameter) × 4mm (inner
135 diameter), was packed with lysozyme crystals. Anhydrous nitrogen gas was passed through the
136 packed column at a flow rate of 10 ml/min, 30 °C and zero relative humidity for 10 days.

137

138 *2.2.3. Preparation of milled dried lysozyme powders*

139

140 Milling was achieved by rotating a marble pestle over the powder within a marble mortar
141 at ~45 cycles per minute (cpm). Milling times of 3, 10, 20, 30, 45, and 60 min were used to produce
142 different samples of milled dried crystals, named 3M, 10M, 20M, 30M, 45M, and 60M,
143 respectively. Another two batches of 3M, 10M, 20M and 60M were also prepared for reference.

144

145 *2.2.4. Preparation of amorphous lysozyme powders with different salt content*

146

147 Precipitated samples were also prepared to explain the effect of NaCl on thermal behaviour
148 of lysozyme particles. These amorphous samples were prepared using the same principle of batch
149 crystallization method. Hence solutions containing 2 % w/v lysozyme plus different amount of
150 NaCl (0, 0.096, 0.16, and 0.8 % w/v) in deionised water were dried under vacuum at a temperature
151 30 °C for two days to produce lysozyme powders theoretically containing 0, 24, 40 and 200 NaCl
152 molecules for each lysozyme molecule, respectively. These four lysozyme samples were named
153 P0, P24, P40, and P200, respectively, and their amorphous nature was confirmed by XRPD.

154

155 *2.2.5. Preparation of lysozyme mixtures from two different samples*

156

157 Unprocessed lysozyme and the 3M sample were mixed at different ratios (w/w) of 3:7, 5:5,
158 and 7:3 in 100 mg samples. The mixtures were lightly mixed in a mortar with a spatula for 10 min
159 and then in a small plastic bag for 10 min to ensure their homogeneity.

160

161 2.3. *Microscopy*

162

163 A Zeiss Axioplan2 polarizing microscope (Carl Zeiss Vision GmbH; Hallbergmoos,
164 Germany) was used to visualize the samples. The accompanying software (Axio Vision 4.2) was
165 then used to determine the projected area diameters of the powders.

166

167 2.4. *Thermogravimetric analysis (TGA)*

168

169 The water content of each sample was estimated using Thermo Gravimetric Analysis (TGA
170 7 Perkin Elmer Ltd., Beaconsfield, UK). Samples of 3-10 mg were heated from 30 °C to 210 °C
171 at a scan rate of 10 °C/min in aluminium pan under nitrogen flow at 20 ml/min. Each sample was
172 analysed in triplicate. The decrease in the weight before decomposition was calculated and was
173 considered as water content. TGA results were validated by re-analyzing the water content of some
174 samples using Karl Fischer Titration (KFT) (701 KF Titrino with 703 Ti stand, Metrohm,
175 Switzerland). Using TGA instead of KFT is that only a few mg is enough for TGA.

176

177 2.5. *Powder X-ray diffraction (PXRD)*

178

179 X-ray powder diffraction patterns of the powders were obtained using a Siemens D5000
180 diffractometer (Siemens, Karlsruhe, Germany), using CuK α radiation ($\lambda = 1.5418\text{\AA}$). The
181 generator was set to 40 kV and 30 mA. Samples were placed into plastic sample holder with zero
182 background and levelled using a glass cover slide. Samples were scanned over an angular range of

183 2-10° (2θ), with a step size of 0.001° and a count time of 3 s per step. The sample stage was spun
184 at 30 rpm. The instrument was calibrated prior to use, using a silicon standard.

185

186 2.6. Differential scanning calorimetry (DSC)

187

188 Differential scanning calorimetry (DSC) thermograms were obtained using a Perkin-Elmer
189 Series 7 DSC (Perkin-Elmer Ltd., Beaconsfield, UK). Samples (4-7 mg) were sealed in aluminium
190 pans. The escape of water was facilitated by placing a pinhole in the lid prior to sealing. The
191 samples were equilibrated at 30 °C and heated to 210 °C at a scan rate of 10 °C/min under a flow
192 of anhydrous nitrogen (20 ml/min). Each sample was analysed in triplicate. The temperature axis
193 and cell constant of the DSC cell were calibrated with indium (10 mg, 99.999 % pure, melting
194 point 156.60 °C, and heat of fusion 28.40 J/g).

195

196 2.6.1. Evaluation of microcalorimetric data

197

198 T_m and calorimetric transition enthalpies (ΔH_{Cal}) were measured by DSC, and then the
199 cooperative unit size (n') was calculated after baseline correction using Eq. (1):

$$200 \quad n' = \Delta H_{vH} / \Delta H_{Cal} \quad (1)$$

201 where ΔH_{vH} is the corresponding van't Hoff enthalpy of the unfolding. Bammel et al. [20]
202 indicated that ΔH_{vH} can be obtained from:

$$203 \quad \Delta H_{vH} = 4RT_m^2 \Delta C_p(\max) / \Delta H_{Cal} \quad (2)$$

204 where R is the universal gas constant ($8.314 \times 10^{-3} \text{ kJ K}^{-1} \text{ mol}^{-1}$), $\Delta C_p(\max)$ is the maximum heat
205 capacity and it is calculated from:

206
$$\Delta C_p(\text{max}) = \text{peak height} / (\text{scan rate} \times \text{sample weight}) \quad (3).$$

207

208 *2.7. FT-Raman spectroscopy*

209

210 FT-Raman spectra of samples were recorded with a Bruker IFS66 optics system using a
211 Bruker FRA 106 Raman module. The excitation source was an Nd: YAG laser operating at 1064
212 nm and a laser power of 50 mW was used. The FT-Raman module is equipped with a liquid
213 nitrogen cooled germanium diode detector with an extended spectrum band width covering the
214 wave number range $1800\text{-}450\text{ cm}^{-1}$. Samples were placed in stainless steel sample cups and
215 scanned 200 times with the resolution set at 8 cm^{-1} . The observed band wave numbers were
216 calibrated against the internal laser frequency and are correct to better than $\pm 1\text{ cm}^{-1}$. The spectra
217 were corrected for instrument response. The experiments were run at a controlled room
218 temperature of $20 \pm 1^\circ\text{C}$.

219

220 *2.8. Enzymatic assay*

221

222 Lysozyme catalyzes the hydrolysis of β -1,4-glycosidic linkages of cell-wall
223 mucopolysaccharides [18]. This principle was used to measure the activity of lysozyme as follows.
224 30 μl of lysozyme solution 0.05 % in phosphate buffer (pH = 5.2; 10 mM) was added to 2.97 ml
225 substrate bacterial suspension 0.025 % of *Micrococcus lysodeikticus* in phosphate buffer (pH =
226 6.24; 66mM). The decrease in the absorption at 450 nm was monitored by using a UV-Vis
227 spectrophotometer (Pu 8700, Philips, UK). The activity was determined by measuring the decrease
228 in the substrate bacterial suspension concentration with time. Hence the slope of the reduction in

229 the light absorption at 450 nm against the time of 3 min, starting when the protein solutions were
230 mixed with the substrate bacterial suspension, was considered to be the indicator of the activity
231 [21]. The measurements were performed in controlled temperature room at 20 °C to avoid
232 fluctuation of lysozyme activity. The concentrations of the protein solutions had been determined
233 prior to the activity tests using Eq. (4).

$$234 \quad [\text{Protein}] = Abs_{280nm} / E_{280nm} \quad (4)$$

235 where [protein] is the concentration of protein in the tested solution w/v%, Abs_{280nm} is the
236 absorption of the tested protein solution at 280nm, E_{280nm} is the absorption of protein standard
237 solution with concentration 0.05 w/v%. The concentrations of the solutions were diluted to be
238 about 0.05 % w/v so as to give absorption of less than 0.8. The activities of all samples were
239 measured relative to that of a corresponding fresh sample, which was considered as the standard
240 solution.

241

242

243 **3. Results and discussion**

244

245 *3.1. Microscopy*

246

247 Fig. S1 shows the photomicrograph of a lysozyme crystal. The anisotropic crystals
248 exhibited a birefringence phenomenon when viewed under a polarizing microscope. The elongated
249 prism-like crystals were in general of a projected area diameter of ~1200 μm (Fig. S1). Their shape

250 was consistent with their preparation temperature, which was 20 °C. The crystal shape of lysozyme
251 is known to be affected by temperature, and a temperature of ~20 °C usually results in elongated
252 prism-like crystals [22]. Samples 3M, 10M, 20M, and 30M had diameters of ~80 μm (Fig. S2), ~7
253 μm (Fig. S3), ~2.5 μm (Fig. S4), and less than 1 μm (Fig. S5), respectively.

254

255 3.2. Thermogravimetric analysis (TGA)

256

257 The results of TGA analysis were used to estimate the water content of the crystal forms.
258 The thermograms (Fig. 1) indicate that on drying using the conditions described above, the water
259 content decreased from 17.3±1.0 % w/w to 2.6±0.3 % w/w. The obtained values of water content
260 in protein powders using TGA were previously shown to be consistent with Karl Fischer titration
261 data [13].

262

263 3.3. X-ray powder diffraction (XRPD)

264

265 The diffractogram presented in Fig. 2A shows an absence of diffraction peaks for the
266 unprocessed lysozyme powder indicating that it was amorphous. However, in Fig. 2B diffraction
267 peaks for the lysozyme crystals at 2θ angles less than 8° are present. Proteins are large molecules
268 and are crystallized typically in unit cells having high d values, and so according to Bragg's law,
269 lysozyme crystals are expected to diffract x-ray at low 2θ angles.

270 Because lysozyme crystals exist in different forms, the CMPR program (Version 1.32) [23]
271 was employed for phase identification. The observed PXRD peaks of the crystals at 2.82°, 3.56°,
272 4.47°, 5.21°, 6.09°, and 6.97° (Fig. 2B) fitted the faces (H,K,L) {(1,1,1), (3,1,0), (4,0,0), (4,1,1),

273 (4,3,1), (5,3,1)} of the tetragonal form of lysozyme with parameters $A=78.54$, $B=78.54$ and
274 $C=37.77$, $\alpha = \beta = \gamma = 90^\circ$ taken from protein data bank reference number 193L [24].

275 Dried lysozyme crystals were also characterised using PXRD and data indicated that the
276 degree of crystallinity was predominantly maintained. The fact that the intensity of the XRPD
277 peaks, which did not notably change (Fig. 2C), was evidence to this effect. Minor up shifting in
278 the diffracted peaks after drying was noticed. This can be due to shrinkage of the unit cell to lower
279 d values upon dehydration. The cell volume of tetragonal lysozyme crystals shrunk under the effect
280 of pressure without a loss in its diffraction property [25]. Our XRPD finding agrees with previous
281 results. For example, both monoclinic and triclinic crystals of HEWL have been shown to maintain
282 their ability to diffract X-rays after dehydration [26] and [27], respectively. However, it contradicts
283 some previous low frequency Raman spectra results, which showed that tetragonal lysozyme
284 crystals resulted in crystallinity loss and conversion into an isotropic material (amorphous) after
285 drying from ~ 33.5 % w/w to ~ 9 % w/w water content by equilibrating at $\sim 30\%$ r.h. [28,29]. In
286 general, although dehydration firstly transfers a protein crystal to a metastable state, which then
287 collapses and loses its packing structure, some crystals survive their crystallinity upon dehydration
288 [26]. The findings of the present study would suggest that the conditions of the drying process
289 would play an important part as to whether crystallinity is maintained. Thus if protein crystals are
290 to be used as a drug delivery vehicle after bulk crystallisation then the subsequent milling and
291 drying conditions will require close control and monitoring to obtain consistent results.

292 Milling of the dried crystals did produce a loss in crystallinity. XRPD was able to follow
293 the loss of crystallinity of dried lysozyme crystals with comminution time. In case of 3M, the three
294 minutes of milling did not induce crystallinity loss (Fig. 2D) as indicated by PXRD. However, the
295 intensity of the diffracted peaks of sample 10M decreased (Fig. 2E). This indicates that lysozyme

296 crystals became partially disordered after only 10 minutes of milling. Fig. 2F shows that 20
297 minutes of milling was sufficient to produce a diffractogram with no clear peaks for the 20M
298 sample, and so its XRPD spectrum became similar to that of the amorphous lysozyme
299 (unprocessed) (Fig. 2A) which is consistent with transformation to the amorphous state.

300

301 *3.4. Differential scanning calorimetry (DSC)*

302

303 Lysozyme DSC thermograms, in the solid state, typically show two endothermic peaks.
304 The broad peak ranging from ~30 to ~140 °C is due to water removal, and its area depends on the
305 water residue in the samples. The second peak at ~200 °C, and its peak maximum was considered
306 to reflect the apparent denaturation temperature (T_m) [13,14,18,19,30]. Fig. 3A shows a typical
307 lysozyme thermogram demonstrating the presence of peaks at similar positions to those mentioned
308 above. Thermograms for all other samples show a consistent water removal peak. However, the
309 position and magnitude of T_m peak was found to depend on the solid state form of the lysozyme
310 powder. The differences observed from DSC analysis align with the differences between the states
311 previously observed by XRPD (Fig. 2). Amorphous lysozyme obtained as received (unprocessed
312 lysozyme) thermally peaked at a T_m of about ~201 °C (Fig. 3A), while samples of crystalline
313 lysozyme (crystals, dried crystals, or the minimally milled 3M sample) gave a lower T_m of about
314 ~187 °C (Figs 4B, 4C and 4D). Evidence that the 10M sample was transformed partially to an
315 amorphous form was apparent from inspection of the DSC thermal scan since it contained two
316 distinct T_m 's consistent with amorphous and crystalline lysozyme forms respectively at separate
317 T_m of ~187 and ~201 °C (Fig. 3E). Similar to the XPRD evidence above, DSC data suggested that

318 further milling completed the amorphous transformation, with the 20M sample being amorphous,
319 and hence it only had the T_m of an amorphous state at $\sim 201^\circ\text{C}$ (Fig. 3F).

320 Close scrutiny of the thermograms showed that for sample 20M (amorphous lysozyme),
321 T_m was slightly decreased by only $\sim 1^\circ\text{C}$ compared to that of the other amorphous lysozyme
322 (unprocessed sample). However, this small difference was significant (t-Test: $P < 0.05$) (Table 1).
323 This minor reduction is likely to be due to the presence of NaCl content in the former sample. The
324 amount of NaCl in the lysozyme crystal is approximated to be ~ 10 NaCl molecules for each
325 lysozyme molecule. At the preparation pH, each molecule of lysozyme needed $\sim 10 \text{Cl}^-$ ions of
326 NaCl to shield its positive charges, which induce the repulsion between lysozyme molecules. This
327 shielding by counter ions of the precipitant (NaCl) is necessary to start nucleation and form crystals
328 in which these ions are trapped [31]. The precipitated samples which were precipitated with
329 different salt contents (P0, P24, P40, and P200) revealed a strong inverse relationship between salt
330 content and T_m (correlation coefficient $r = -0.92$). The values of T_m were 202.2 ± 0.3 , 200.3 ± 0.5 ,
331 198.2 ± 0.5 , and 195.8 ± 0.2 , respectively, and their DSC profiles and amorphous PXRD patterns are
332 provided in supplementary data (Fig. S6 and Fig. S7). Therefore, according to this correlation, the
333 presence of around 10 NaCl molecules for each lysozyme molecule reduces T_m by $\sim 1^\circ\text{C}$.

334 The T_m of a protein does not necessarily represent a solid-liquid transformation. Proteins
335 do not melt, but they change their molecular conformation from a native to denatured state at the
336 T_m which overcomes the attractive intra-molecular forces, which preserve their native state. In
337 solution state where lysozyme molecules are well separated from each other by water molecules,
338 the T_m of lysozyme is $\sim 76^\circ\text{C}$ [32]. The cooperative unit (n') is typically thought to estimate the
339 lowest number of molecules which form an independently melting cluster of molecules within a
340 sample [33] and is used as an indicator of the degree of unfolding cooperativity of lysozyme [15].

341 In solution state, n' of lysozyme solutions ranges from 1 to 2 (i.e., ~1.5) [34] Compared with the
342 solution state, n' of lysozyme in the dried amorphous form (e.g. unprocessed or 20M sample) was
343 estimated to increase from ~1.5 to ~10.5, and this is associated with the increase in T_m from ~76
344 to ~201°C (Table 1).

345 We can postulate that when the cooperative unit increases, a higher temperature is needed
346 to unfold the lysozyme molecular aggregates, and that the larger the cooperative unit, the higher
347 the T_m . The n' of the crystalline form (e.g. crystals, dried crystals, 3M) was lower than those of the
348 amorphous form by ~2.5 unit (t-Test: $P < 0.05$) (Table 1). This explains the reduction in T_m of the
349 crystalline form by ~14 °C compared to the amorphous form. Although the unfolding of proteins
350 is an intra-molecular phenomenon rather than an inter-molecular phenomenon, the unfolding of a
351 molecule within a molecular aggregate is resisted by the steric hindrance and repulsion of the other
352 molecules in the unit.

353 Table 1 and Fig. 3 show that milling decreased the ΔH_{Cal} of the unfolding peak of the
354 crystalline form (ΔH_{Cal}^{Cr}) with a corresponding increase in the ΔH_{Cal} of the unfolding peak of the
355 amorphous form (ΔH_{Cal}^{Am}). When ΔH_{Cal}^{Cr} vanished, ΔH_{Cal}^{Am} reached a maximum of 105.9 kJ.mol⁻¹
356 (as in the case of 20M sample), which is lower than those of both the crystalline form (dried
357 crystals) and the amorphous form (unprocessed sample). Therefore, the complete crystalline-
358 amorphous transformation associated with slightly mechanical denaturation (~17%). The further
359 milling denatured gradually the amorphous form, as ΔH_{Cal}^{Am} decreased gradually by milling (i.e.,
360 30M and 45M samples). Prolonged milling of dried crystals produced thermal evidence of a loss
361 of unfolding due to the absence of a T_m as in the case of 60M (Fig. 3I). Fig. 4 summarizes the
362 correlation between the milling time and the calorimetric unfolding enthalpies of the crystalline
363 and amorphous peaks. This observation coupled with the milling data discussed above is

364 suggestive that milling initially induced a partial crystalline-amorphous transformation, followed
365 by a complete amorphization transformation, and then on subsequent prolonged milling produced
366 complete mechanical denaturation. Although a small part of lysozyme molecules denatured during
367 the crystalline-amorphous transformation, the mechanical denaturation of crystalline lysozyme
368 mainly goes through amorphous state. Similarly, the other two batches of 3M, 10M, 20M and 60M
369 verified the crystalline-amorphous-denatured transformations of lysozyme powders during milling
370 and the distinctive thermal behaviours of each form (Table 2).

371

372 *3.4.1. Quantitative analysis of lysozyme solid states by DSC*

373

374 Conventional DSC has been used to estimate the different solid phases in lactose powders.
375 The estimation depended on knowing the enthalpy of solid phase transformation for each form.
376 This DSC approach has been previously verified [35].

377 Applying the same approach to monitor the processing of the lysozyme powders,
378 crystalline lysozyme (i.e. crystals) and amorphous lysozyme (i.e. unprocessed powder) produced
379 similar enthalpies (t-Test: $P > 0.05$), with an average of 125 and 134 $\text{kJ}\cdot\text{mol}^{-1}$, respectively.
380 Therefore, these values were considered as the enthalpy of the native solid lysozyme. We assume
381 that milled solid lysozyme powders would consist of mixtures of crystalline, amorphous and/or
382 denatured lysozyme. The percentages of crystalline form (Cr%), amorphous form (Am%), and
383 denatured form (De%) in the lysozyme powders can be determined from:

$$384 \quad \text{Cr\%} = (\Delta H_{\text{Cal}}^{\sim 187} / 125) \times 100 \quad (5)$$

$$385 \quad \text{Am\%} = (\Delta H_{\text{Cal}}^{\sim 201} / 134) \times 100 \quad (6)$$

$$386 \quad \text{De\%} = 100 - (\text{Cr\%} + \text{Am\%}) \quad (7)$$

387 where $\Delta H_{Cal}^{\sim 187}$ and $\Delta H_{Cal}^{\sim 201}$ are the measured enthalpy ($\text{kJ}\cdot\text{mol}^{-1}$) of the unfolding peaks at T_m of
388 ~ 187 and ~ 201 °C, respectively.

389 To test the quantitative hypothesis amorphous lysozyme (unprocessed sample) and
390 crystalline lysozyme (3M sample) were mixed at different ratios of 3:7, 5:5, and 7:3 and then they
391 analysed by DSC (same procedures). According to Eq. (5), (6) and (7), the amorphous sample
392 contains 100% native lysozyme, but the crystalline sample, which had $\Delta H_{Cal}^{\sim 187} = 114.4 \text{ kJ}\cdot\text{mol}^{-1}$,
393 contains 91.5% native and 8.5% denature lysozyme. Therefore, the three mixtures of
394 unprocessed:3M (3:7, 5:5 and 7:3) should contain (27.4%, 70.0%, 2.6%), (45.7%, 50.0%, 4.3%)
395 and (64.0%, 30.0%, 6.0%) of (Cr%, Am%, and De%), respectively. These calculated crystalline,
396 amorphous and denatured percentages using the above equations were similar to the actual
397 percentages in the mixtures listed in Table 3. Fig. 5 shows the distinctive unfolding peaks of the
398 crystalline and amorphous forms of the mixtures.

399

400 3.5. FT-Raman study

401

402 Raman spectroscopy was used to compare the molecular conformation of crystalline (3M
403 sample), amorphous lysozyme (20M sample) and extensively milled (denatured) lysozyme (60M
404 sample) with dried lysozyme crystals. The band at 1447 cm^{-1} indicates the CH bending vibrations
405 of aliphatic side chains, and its intensity and position are unaffected by changes induced in protein
406 structure after dehydration or applying different stresses [36]. Therefore, it was used as internal
407 intensity standard to normalize Raman spectra before comparison (Fig. 6A).

408 Compared to the amorphous form of lysozyme (20M sample), the crystalline form (dried
409 crystals and 3M sample) showed greater intensity of the ν Ca-C-N mode at 930 cm^{-1} (Fig. 6B),

410 and produced a higher vibration mode of amide III (N-H in-plane bend + C-N stretch) at ~1265
411 cm^{-1} (Fig. 6C) and a lower and sharper vibration mode of amide I (C=O stretch) at ~1660 cm^{-1}
412 (Fig. 6D). The intensity of ν Ca-C-N mode at 930 cm^{-1} [37], upshifting of amide III (N-H in-plane
413 bend + C-N stretch) [38], and downshifting and sharpening of the line of amide I (C=O stretch) at
414 ~1660 cm^{-1} [39] indicates a higher α -helix content. This means that the crystalline form maintained
415 the α -helix structure of native lysozyme more than the amorphous form. The native secondary
416 structure of lysozyme consists of three alpha helix regions extending 5-15, 24-34 and 88-96 amino
417 acid residues [24].

418 Rich α -helix structures have a low tendency to aggregate compared to rich β -sheet
419 structures [15]. This possibly explains why the crystalline form had less tendency to thermally
420 unfold in lower n' compared to the amorphous form. Therefore, according to our above postulation,
421 the spectroscopic observation that the crystalline form contains more alpha helix may explain why
422 the crystalline lysozyme unfolded at lower T_m .

423 Comparison of the spectrum of the denatured lysozyme (sample 60M) with that of the
424 amorphous form shows that mechanical denaturation induced further reduction in the intensity of
425 ν Ca-C-N mode at 930 cm^{-1} (Fig. 6B), which in turn implies a further reduction in α -helix content.

426

427 3.6. Enzymatic assay

428

429 Interestingly, milled samples did not show a reduction in biological activity (ANOVA: $P >$
430 0.05) (Table 4). The ability of lysozyme to re-nature on dissolution is a possible explanation for
431 this finding. Indeed, previous research has shown the strong refolding ability of lysozyme upon
432 dissolution in aqueous media [40]. Likewise, despite significant loss of the Raman vibrations of

433 lysozyme upon denaturation by γ -irradiation, the denatured lysozyme samples almost fully
434 recovered their biological activity on dissolution [39].

435

436 **4. Conclusions**

437

438 Bulk crystallisation of protein therapeutic molecules for controlled drug delivery studies is
439 of interest to the biopharmaceutical industry. The complexity of biotherapeutic molecules is likely
440 to lead to complex material properties of crystals in the solid state. Here we exemplify, using the
441 model drug lysozyme, the effects of processing lysozyme crystals and are able to show distinct
442 differences in the properties of the materials after processing which could be used in optimising
443 and controlling processes for the purposes of quality by design.

444 By drying lysozyme crystals using a controlled method we were able to evidence the
445 removal of water and maintain crystallinity. However, the size reduction of dried lysozyme crystals
446 by milling was shown to promote formation of an amorphous solid-state form. Raman
447 spectroscopy provided evidence that the amorphous form was then denatured in the solid state by
448 further milling. The milling time was the critical attribute determining the extent of the
449 transformations. DSC was successfully employed in monitoring the three different states of
450 lysozyme (namely crystalline, amorphous, and denatured) in the solid state. The DSC thermogram
451 of the crystalline lysozyme exhibited T_m at ~ 187 °C which was lower than that of amorphous
452 lysozyme by ~ 14 °C. The mechanically denatured lysozyme did not provide a thermal unfolding
453 transition. The calorimetric enthalpies of the crystalline and amorphous peaks were used to analyze
454 quantitatively the three different states of lysozyme. XRPD data were consistent with the
455 crystallinity of lysozyme identified by DSC. Interpretation of the Raman data from the same

456 samples is consistent with a crystalline form having a lower tendency to aggregate due to its greater
457 α -helix rich structure compared to the amorphous form. Significantly, although the molecular
458 arrangement and molecular conformation of lysozyme changed during milling, its biological
459 activity did not decrease. Clearly, subtle changes in solid-state processing conditions of crystalline
460 lysozyme can bring about major changes in its solid-state properties. The effects of a wider range
461 of milling variables, including different mill types, on lysozyme solid-state transformations and
462 behaviour will be the subject of a future study.

463

464

465 **Acknowledgements**

466

467 The authors thank Dr Ian S. Blagbrough (University of Bath) for helpful discussions and
468 Dr Colin Seaton (University of Bradford) for help in using the CMPR program. MAM gratefully
469 acknowledges CARA (Zeid Al-Bayaty and Kate Robertson) for providing an academic fellowship.

470

471

472

473

474

475

476

477

478

479

480

481

482

483

484

485

486

487 **References**

- 488 [1] W. Zhao, R. Yang, R. Lu, Y. Tang, W. Zhang, Investigation of the mechanisms of pulsed
489 electric fields on inactivation of enzyme: lysozyme, *J. Agric. Food Chem.* 55 (2007) 9850-
490 9858.
- 491 [2] E.A. Charter, G. Lagarde, Natural anti-microbial systems: Lysozyme and other proteins in
492 eggs, in: C.A. Batt (Second Edition), *Encyclopedia of Food Microbiology*, Elsevier, Ltd., San
493 Diego, 2014, pp. 936-940.
- 494 [3] M.I. Hoq, H.R. Ibrahim, Potent antimicrobial action of triclosan–lysozyme complex against
495 skin pathogens mediated through drug-targeted delivery mechanism, *Eur. J. Pharm. Sci.* 42
496 (2011) 130-137.
- 497 [4] D. Cerven, G. DeGeorge, D. Bethell, 28-Day repeated dose oral toxicity of recombinant human
498 apo-lactoferrin or recombinant human lysozyme in rats, *Regul. Toxicol. Pharm.* 51 (2008) 162-
499 167.
- 500 [5] H.J. Cho, P. Balakrishnan, S.J. Chung, C.K. Shim, D.D. Kim, Evaluation of protein stability
501 and in vitro permeation of lyophilized polysaccharides-based microparticles for intranasal
502 protein delivery, *Int. J. Pharm.* 416 (2011) 77-84.
- 503 [6] O.N. Ógáin, J. Li, L. Tajber, O.I. Corrigan, A.M. Healy, Particle engineering of materials for
504 oral inhalation by dry powder inhalers. I-Particles of sugar excipients (trehalose and raffinose)
505 for protein delivery, *Int. J. Pharm.* 405 (2011) 23-35.
- 506 [7] B. Zhou, Y. Li, H. Deng, Y. Hu, B. Li, Antibacterial multilayer films fabricated by layer-by-
507 layer immobilizing lysozyme and gold nanoparticles on nanofibers, *Colloids Surf. B*
508 *Biointerfaces* 116 (2014) 432-438.
- 509 [8] G. Sax, G. Winter, Mechanistic studies on the release of lysozyme from twin-screw extruded
510 lipid implants, *J. Control. Release* 163 (2012) 187-194.
- 511 [9] W. Schlocker, S. Gschließer, A. Bernkop-Schnürch, Evaluation of the potential of air jet
512 milling of solid protein-poly (acrylate) complexes for microparticle preparation, *Eur. J. Pharm.*
513 *Biopharm.* 62 (2006) 260-266.
- 514 [10] H. Hoyer, W. Schlocker, K. Krum, A. Bernkop-Schnürch, Preparation and evaluation of
515 microparticles from thiolated polymers via air jet milling, *Eur. J. Pharm. Biopharm.* 69 (2008)
516 476-485.
- 517 [11] J.H. Kwon, C.W. Kim, A novel insulin microcrystals preparation using a seed zone method,
518 *J. Cryst. Growth* 263 (2004) 536-543.
- 519 [12] C. Müller, J. Ulrich, The dissolution phenomenon of lysozyme crystals, *Cryst. Res. Technol.*
520 47 (2012) 169-174.
- 521 [13] A.A. Elkordy, R.T. Forbes, B.W. Barry, Integrity of crystalline lysozyme exceeds that of a
522 spray-dried form, *Int. J. Pharm.* 247 (2002) 79-90.
- 523 [14] A.A. Elkordy, R.T. Forbes, B.W. Barry, Stability of crystallised and spray-dried lysozyme,
524 *Int. J. Pharm.* 278 (2004) 209-219.
- 525 [15] H. Liu, P. Yin, S. He, Z. Sun, Y. Tao, Y. Huang, H. Zhuang, G. Zhang, S. Wei, ATP-induced
526 noncooperative thermal unfolding of hen lysozyme, *Biochem. Biophys. Res. Commun.* 397
527 (2010) 598-602.
- 528 [16] A. Badkar, P. Yohannes, A. Banga, Application of TZERO calibrated modulated temperature
529 differential scanning calorimetry to characterize model protein formulations, *Int. J. Pharm.* 309
530 (2006) 146-156.

- 531 [17] Y. Hirakura, H. Yamaguchi, M. Mizuno, H. Miyanishi, S. Ueda, S. Kitamura, Detection of
532 lot-to-lot variations in the amorphous microstructure of lyophilized protein formulations
533 International, *Int. J. Pharm.* 340 (2007) 34-41.
- 534 [18] R.R. Haj-Ahmad, A.A. Elkordy, C.S. Chaw, A. Moore, Compare and contrast the effects of
535 surfactants (Pluronic®F-127 and Cremophor®EL) and sugars (β -cyclodextrin and inulin) on
536 properties of spray dried and crystallised lysozyme, *Eur. J. Pharm. Sci.* 49 (2013) 519-534.
- 537 [19] Z. Du, Y.X. Guan, S.J. Yao, Z.Q. Zhu, Supercritical fluid assisted atomization introduced by
538 an enhanced mixer for micronization of lysozyme: Particle morphology, size and protein
539 stability, *Int. J. Pharm.* 421 (2011) 258-268.
- 540 [20] B.P. Bammel, D.D. Hamilton, R.P. Haugland, H.P. Hopkins, J. Schuette, W. Szalecki, J.C.
541 Smith, NMR, calorimetric, spin-label, and optical studies on a trifluoromethyl-substituted
542 styryl molecular probe in dimyristoylphosphatidylcholine vesicles and multilamellar
543 suspensions: a model for location of optical probes, *BBA-Biomembranes* 1024 (1990) 61-81.
- 544 [21] D. Shugar, Measurement of lysozyme activity and the ultraviolet inactivation of lysozyme,
545 *Biochim. Biophys. Acta* 8 (1952) 302.
- 546 [22] J. Lu, X.J. Wang, C.B. Ching, Batch crystallization of soluble proteins: effect of precipitant,
547 temperature and additive, *Prog. Cryst. Growth Ch.* 45 (2002) 201-217.
- 548 [23] B.H. Toby, CMPR - a powder diffraction toolkit, *J. Appl. Crystallogr.* 38 (2005) 1040-1041.
- 549 [24] M.C. Vaney, S. Maignan, M. RiesKautt, A. Ducruix, High resolution structure (1.33
550 angstrom) of a HEW lysozyme tetragonal crystal grown in the APCF apparatus: data and
551 structural comparison with a crystal grown under microgravity from SpaceHab-01 mission,
552 *Acta Crystallogr. D Biol. Crystallogr.* 52 (1996) 505-517.
- 553 [25] C. Charron, M.C. Robert, B. Capelle, A. Kadri, G. Jenner, R. Giegé, B. Lorber, X-ray
554 diffraction properties of protein crystals prepared in agarose gel under hydrostatic pressure, *J.*
555 *Cryst. Growth* 245 (2002) 321-333.
- 556 [26] K. Harata, T. Akiba, Structural phase transition of monoclinic crystals of hen egg-white
557 lysozyme, *Acta Crystallogr. D Biol. Crystallogr.* 62 (2006) 375-382.
- 558 [27] G.S. Kachalova, V.N. Morozov, T.Y. Morozova, E.T. Myachin, A.A. Vagin, B.V.
559 Strokopytov, Y.V. Nekrasov, Comparison of structures of dry and wet hen egg-white lysozyme
560 molecule at 1.8 Å resolution, *FEBS Lett.* 284 (1991) 91-94.
- 561 [28] H. Urabe, Y. Sugawara, M. Ataka, A. Rupprecht, Low-frequency Raman spectra of lysozyme
562 crystals and oriented DNA films: Dynamics of crystal water, *Biophys. J.* 74 (1998) 1533-1540.
- 563 [29] A.B. Kudryavtsev, G. Christopher, C.D. Smith, S.B. Mirov, W.M. Rosenblum, L.J. DeLucas,
564 The effect of ordering of internal water in thaumatin and lysozyme crystals as revealed by
565 Raman method, *J. Cryst. Growth* 219 (2000) 102-114.
- 566 [30] W.L. Hulse, R.T. Forbes, M.C. Bonner, M. Getrost, Do co-spray dried excipients offer better
567 lysozyme stabilisation than single excipients?, *Eur. J. Pharm. Sci.* 33 (2008) 294-305.
- 568 [31] F. Rosenberger, Protein crystallization, *J. Cryst. Growth* 166 (1996) 40-54.
- 569 [32] A.A. Elkordy, R.T. Forbes, B.W. Barry, Study of protein conformational stability and
570 integrity using calorimetry and FT-Raman spectroscopy correlated with enzymatic activity,
571 *Eur. J. Pharm. Sci.* 33 (2008) 177-190.
- 572 [33] H. Lu, J.C. Wojtowicz, I.A. Butovich, Differential scanning calorimetric evaluation of human
573 meibomian gland secretions and model lipid mixtures: Transition temperatures and
574 cooperativity of melting, *Chem. Phys. Lipids* 170 (2013) 55-64.

- 575 [34] N. Hassan, P.V. Verdes, J.M. Ruso, Assessment of interactions between four proteins and
576 benzothiazole derivatives by DSC and CD, *J. Chem. Thermodyn.* 43 (2011) 399-404.
- 577 [35] W. Kaialy, G.P. Martin, M.D. Ticehurst, P. Royall, M.A. Mohammad, J. Murphy, A.
578 Nokhodchi, Characterisation and deposition studies of recrystallised lactose from binary
579 mixtures of ethanol/butanol for improved drug delivery from dry powder inhalers, *AAPS J.* 13
580 (2011) 30-43.
- 581 [36] T.J. Yu, J.L. Lippert, W.L. Peticolas, Laser Raman studies of conformational variations of
582 poly-L-lysine, *Biopolymers* 12 (1973) 2161-2176.
- 583 [37] S. Ikeda, Heat-induced gelation of whey proteins observed by rheology, atomic force
584 microscopy, and Raman scattering spectroscopy, *Food Hydrocoll.* 17 (2003) 399-406.
- 585 [38] E.C. Li-Chan, The applications of Raman spectroscopy in food science, *Trends Food Sci.*
586 *Tech.* 7 (1996) 361-370.
- 587 [39] A. Torreggiani, M. Tamba, I. Manco, M.R. Faraone-Mennella, C. Ferreri, C. Chatgililoglu,
588 Radiation damage of lysozyme in a biomimetic model: some insights by Raman spectroscopy,
589 *J. Mol. Struct.* 744 (2005) 767-773.
- 590 [40] C. Pérez-Rodríguez, N. Montano, K. Gonzalez, K. Griebenow, Stabilization of α -
591 chymotrypsin at the CH_2Cl_2 /water interface and upon water-in-oil-in-water encapsulation in
592 PLGA microspheres, *J. Control. Release* 89 (2003) 71-85.
- 593
- 594