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Mapping the solid-state properties of crystalline lysozyme during pharmaceutical unit-operations

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ABSTRACT

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

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1. Introduction

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

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

Differential scanning calorimetry (DSC) is a well-established and widely used technique to monitor solid state transformations.

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

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

2. Materials and methods

2.1. Materials

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

2.2. Sample preparation

2.2.1. Preparation lysozyme crystals using batch crystallization method

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

2.2.2. Preparation of dried lysozyme crystals

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

2.2.3. Preparation of milled dried lysozyme powders

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

2.2.4. Preparation of amorphous lysozyme powders with different salt content

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

2.2.5. Preparation of lysozyme mixtures from two different samples

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

2.3. Microscopy

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

2.4. Thermogravimetric analysis (TGA)

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

2.5. Powder X-ray diffraction (PXRD)

X-ray powder diffraction patterns of the powders were obtained using a Siemens D5000 diffractometer (Siemens, Karlsruhe, Germany), using Cu*K*α radiation (λ = 1.5418 Å). The generator was set to 40 kV and 30 mA. Samples were placed into plastic sample holder with zero background and levelled using a glass cover slide. Samples were scanned over an angular range of 2–10° (2θ), with a step size of 0.001° and a count time of 3 s per step. The sample stage was spun at 30 rpm. The instrument was calibrated prior to use, using a silicon standard.

2.6. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) thermograms were obtained using a Perkin-Elmer Series 7 DSC (Perkin-Elmer Ltd., Beaconsfield, UK). Samples (4–7 mg) were sealed in aluminium pans. The escape of water was facilitated by placing a pinhole in the lid prior to sealing. The samples were equilibrated at 30 °C and heated to 210 °C at a scan rate of 10 °C/min under a flow of anhydrous nitrogen (20 ml/min). Each sample was analysed in triplicate. The

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