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Study on the influence of lysozyme crystallization conditions on crystal properties in crystallizers of varied sizes when temperature is the manipulated variable

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ABSTRACT

In this work, crystallization experiments were conducted in three different sizes of crystallizers (5 and 100 ml, and 1 L) to study the influence of temperature on the crystallization of lysozyme. Lysozyme solutions with concentrations of 40 and 30 g L^{-1} and 10% (w/w) NaCl were used. The temperature was reduced from 20 to 0 °C with various cooling rate and stirring speed. The data indicated that crystallization with cooling but without agitation or with agitation but without cooling led to low yield and inconstancy between batches, whereas that with combined cooling and agitation resulted in tetragonal crystals with high yields. Parameters, including crystallization onset, crystal morphology, crystal size distribution, concentration, supersaturation, and yield were examined by *in situ* and *ex situ* observations. The observations within small cooling rate range of 0.030–0.111 °C min^{-1} indicated that minor changes in cooling rate could cause significant differences in these parameters. The comparison with thermostatic experiment showed that cooling could cause the crystal sizes to be widely dispersed. While high cooling rate lead shorter crystallization onset time and higher supersaturation, thereby result in larger crystal size, higher tendency of aggregation and wider crystal size distribution, low cooling rate can pose a great challenge to the temperature control in scale-up crystallization. The work also demonstrated that the crystallization conditions obtained from 5- to 100-ml crystallizers, from which well-defined crystals with high yields were obtained, could successfully be reproduced in 1-L crystallizer.

1. Introduction

In small molecule pharmaceuticals, drugs containing active pharmaceutical ingredients (API) are often in crystalline form. Only small portion of over 240 biopharmaceutical products marketed in 2014 [1] is in crystalline form. Strong evidences have demonstrated that delivery of drugs in crystalline form (rather than in liquids) has numerous advantages. The crystalline form of biopharmaceutical proteins not only has improved bioavailability and stability, but also adjustable solubility and easily controllable release [2]. In addition, compared to the liquid form, the crystalline form often has much longer shelf life, and is easier to handle, transport and store [3–5]. In order to produce crystals with the highest purity, crystallinity, and yield, it is important that the protein crystallization processes and conditions are optimized. Un-optimized operational conditions could lead to amorphous that have poor stability and low purity [3,4,6].

Large-scale production of protein crystals is highly challenging technically. The literatures on protein crystallization are largely about obtaining large single crystals suitable for X-Ray diffraction with the purpose of molecular structure analysis [7–12]. To obtain large single crystals suitable for X-ray diffraction, the crystallization is mainly carried out at a constant temperature in micro-crystallizers, which often is extremely time-consuming with relatively low yields [13]. Moreover, the preparative chromatographic method that are currently and widely used also involves rather cumbersome steps, as has mentioned by some researchers [14]: it is of low efficiency and hardly possible for operation at commercial scale production. However, in technical-scale protein crystallization processes, large single crystals are not essential [15]. Whilst the crystals can be relatively small, the growth rate should be high enough to meet the demands of industrial production and the requirements of GMP (Good Manufacturing Practices). Therefore, in contrast to the preparative chromatographic methods, the technical-

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scale crystallization may only focus on ensuring a low cost while maintaining high efficiency of purification [16].

Researches on scale-up of protein crystallization are still limited. Smejkal et al. [15] reported a study on crystallization of Canabiknmab Fab-Fragment and lysozymes under thermostatic conditions in three different reactor sizes. They found that successful crystallization scale-up, which was conducted thermostatically for 24–72 h, could be achieved when the maximum local energy dissipation is kept constant through different reactor sizes. The same method was applied to thermostatic crystallization of full-length antibodies and fragments in 1-L stirred tanks [17,18]. Nevertheless, the isothermal crystallization is often time-consuming with low yield and reproducibility [16], as has demonstrated in the work of Smejkal et al. [15], in which the crystallization was conducted for 24–72 h. Hebel et al. [19] studied the use of ionic liquids as additives in scale-up protein crystallization, and observed that the crystals had faster growth with higher yield. The introduction of an additive into crystallization is however controversial because it not only requires further edulcoration, but the remaining residues in the solvent channel may also have potential toxicity [18]. Similar observation has also been reported in solvent freeze-out method in protein crystallization, despite the encouraging results obtained from lysozyme purified from lysozyme-ovalbumin mixture, as reported by Diaz Borbon and Ulrich [20]. Maosoongnern et al. [21] conducted seeded isothermal crystallization of lysozyme-ovalbumin mixture in a 100-ml vessel for 24 h and obtained comparable purification results with a yield of 80%. Protein crystallization based on gas diffusion has also been investigated [22–24]; it is however considerably difficult to control and difficult to achieve process scale-up.

Among parameters affecting the crystallization process, temperature can easily be controlled and implemented; it is therefore the preferred parameter chosen in controlling the supersaturation, given that the protein solubility in the solvent significantly varies with temperature. Manipulating temperature via cooling has been proven to be a better option in some protein crystallization studies [25–27], but the method is limited to μL-scale. Based on a study using a 15-μL crystallizer, Astier et al. [25] summarized the advantages of temperature alteration as a crystallization parameter including constant composition, ease of control and monitoring and reversibility. Weber et al. [26] successfully crystallized jack bean urease using the extraction and cooling methods. The crystallization was carried out in the presence of three precipitants in a 50-ml non-agitated vessel for 2 days. The yield and purity were however low.

According to above, the influence of crystallization conditions, including cooling rate, stirring speed, concentration on product quality, and reactor sizes, as well as scalability and repeatability, should be systematically studied. The data obtained may add useful knowledge to the state of the art of protein crystallization. Therefore, in this work, we systematically studied the influence of cooling rates and stirring speed on the crystallization of lysozyme carried out in 5 ml, 100 ml and 1 L batch crystallizers. The morphology, size distribution and concentration of the crystals were characterized by an online imaging system, and the supersaturation profile was analyzed using an *ex situ* observation of sampling solutions.

2. Materials and methods

2.1. Preparation of samples and crystallization solutions

Hen egg white lysozyme powder used in the experiment was purchased from Sigma-Aldrich, Germany (No. 62971). In order to obtain tetragonal crystals, 0.1 M (mol of sodium acetate/1 L of water) sodium acetate buffer solution (titrated to pH = 4.5) was prepared. Lysozyme solutions were prepared by dissolving lysozyme powder (without further purification) in the buffer solution at final concentrations of 10–80 g L^{-1}. Precipitant solutions were prepared by dissolving sodium chloride pellet in the buffer solution at final concentrations of 3.5–25% (w/w). The concentration mentioned below refers to the concentration before mixing with the precipitant solution unless otherwise stated. Prior to use, the solutions were centrifuged at 1300 rpm for 15 min, filtered through 0.22 μm membrane filters, and stored at 20 °C.

2.2. Parallel crystallization experiments

Parallel crystallization experiments were conducted in a sitting drop 24-well Linbro plate. Hen egg white lysozyme (HEWL) and NaCl solutions were mixed in the wells, and 25% (w/w) NaCl solution was placed in each cell as a dispersing agent. The plate was then sealed and incubated at a constant temperature of 20 °C in a constant humidity chamber for 24 h. After that, the samples were examined under a microscope.

2.3. Micro-batch cooling crystallization experiments

For the micro-batch cooling crystallization experiments, feasible concentrations were screened in the above tests. Appropriate concentrations of HEWL and NaCl solutions were mixed to a final volume of 200 μL in a small transparent quartz crucible, which was then covered a clean slide to prevent evaporation. Subsequently, the crucible was placed in hot-stage reactor with high-precision controllable temperature and cooling rate. The investigated cooling rates were ranged from 0.03 to 1 °C min^{-1}. The temperature was reduced from 20 to 0 °C at controlled fixed cooling rates of 0.03–1 °C min^{-1}, thus the cooling duration was between 660 and 10 min. The entire cooling crystallization process was monitored and analyzed under a microscope, whereby the crystal sizes were measured and averaged.

2.4. Agitated batch cooling crystallization

Three geometrically similar stirred tanks with working volumes of 5 ml, 100 ml and 1 L were used in the scale-up crystallization experiments. Flat-jacketed beakers were used as the reaction tanks, and anchor impellers were used for gentle mixing in the vessels. The ratio between heights and vessel filling heights was kept constant at one-third. Hen egg white lysozyme and NaCl solutions were mixed in the tanks, and a temperature probe, which was connected to the recirculation cooler to alter the temperature, was then immersed in the solutions. The specialized temperature control software was used to control the cooling rates, which were fixed within the selected range. The stirring rate, determined by constant tip speed at different operating volumes, was set to a range of 50–250 rpm. The 2D Vision Probe purchased from Pharma Vision (Qingdao) Intelligent Technology Ltd. was used for real-time observation of the crystallization process. However, due to the excessive amount of crystals grown, the observation by 2D Vision Probe was carried out only to examine the crystallization onset, but not to determine the crystal's shapes and sizes. In the *ex situ* determination of crystal size distributions, a sample containing crystallization solution (as well as crystals) was withdrawn using a dropper at various time intervals and undergone two different processes as follows: (i) one part of the sample was placed under a microscope, specially equipped with a camera, thereby the crystal microscopic images were taken. The image frames were managed by 'SHAPE' software provided by Pharma Vision (Qingdao) Intelligent Technology Ltd. The software allows the processing of images, such as edge detection and particle identification, and the pre-processing and segmentation methods, such as contrast adjustment, noise removal, and multi-scale segmentation. After processing, the granule information, e.g. equivalent diameter, granule size distribution and aspect ratio, was exported; and (ii) the other part of the sample was filtered through a 0.22-μm syringe filter, and then diluted by 50 times prior to absorbance measurement at 280 nm by a UV spectrophotometer (UVmini-1240, SHIMADZU Company, Japan), from which its concentration was calculated. After the crystallization was completed, the bulk liquid was filtered through a