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Protein crystal occurrence domains in selective protein crystallisation for bio-separation

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Abstract

Bio-separation is a key bottleneck in the manufacture of biopharmaceuticals. In this work, we report an experimental evidence of direct selective protein crystallisation from a binary protein mixture solution. Lysozyme-thaumatin mixtures with a wide protein composition range (0 – 100 mg/mL, respectively) were tested against the same crystallisation cocktail conditions using hanging-drop vapour-diffusion (HDVD) crystallisation method. This work demonstrates selectivity of crystallisation from a model binary protein mixture and four crystal occurrence domains were determined as the operation windows of selective crystallisation of the target protein: 1) unsaturated region with no crystal formation, 2 & 3) target region with only single type of protein crystals (lysozyme crystals only or thaumatin crystals only) and 3) mixture region in which a mixture of both types of protein. This study demonstrates that protein crystallisation is not only applicable to high-purity protein solution and this also emphases the vital impacts of the presence of protein impurities in the process of target protein crystallisation. The study concludes protein
crystallisation is a feasible approach to separate a target protein from a complex mixture environment which can be achieved by manipulating crystallisation operation conditions such as mixture composition, precipitant concentration, and operation time.

1. Introduction

Therapeutic proteins such as monoclonal antibodies have attracted major interests in the current pharmaceutical market. Progresses in biotechnology in the past decades has resulted in the approval of 285 distinct active biopharmaceutical products which are predominated by monoclonal antibodies and the sales of these products continue to grow reaching a total revenue of $188 billion alone in 2017. Nevertheless, downstream separation processes have now become the bottleneck of cost-effective manufacturing of these protein-based products. Up to 80 % of manufacturing costs attribute to the downstream purification processes which mainly rely on multi-step protein A chromatography technology. In the meanwhile, advances in upstream processes such as cell technology have led to higher titres of secreted proteins which is now beyond 5 g/L, creating greater challenge for protein A chromatography as effective purification steps.

Researchers are seeking alternative technologies to replace or partially substitute conventional chromatography steps towards more lucrative, rapid, and robust downstream separation processes. Various alternative separation technologies have been investigated, including solvent extraction, membrane-based method, precipitation and crystallisation. Crystallisation serves as a common purification
processes of both inorganic and organic small molecule products in numerous conventional chemical industries. Moreover, crystalline protein is believed to have a higher purity and stability which can benefit formulation, storage, and drug delivery steps.\textsuperscript{19-22} Though most of the studies of protein crystallisation focused on obtaining large single crystals for biomolecular structural studies, in the past few decades, researchers have demonstrated that protein crystallisation is also a feasible and scalable purification and isolation technology for downstream bio-separation. Judge et al. demonstrated the feasibility of ovalbumin recovery via bulk crystallisation in 1 L stirred batch crystalliser.\textsuperscript{23} Jacobsen et al. were able to obtain microbial lipase crystals from clarified concentrated fermentation broths.\textsuperscript{24} Hekmat's group has demonstrated that crystallisation is a scalable process by successfully transfer crystallisation of antigen-binding fragment FabC225 from 10 $\mu$L vapour diffusion experiments to a 100 mL batch crystallisation process.\textsuperscript{25} A few continuous crystallisation platforms have also been developed in lab-scale to demonstrate the potential of adapting crystallisation in continuous manufacturing fashion, including stirred classified product removal tank,\textsuperscript{26} tubular plug-flow crystalliser,\textsuperscript{27} continuous crystalliser with oscillatory flow,\textsuperscript{28, 29} and meso oscillatory flow crystalliser.\textsuperscript{30} In most cases, the focus has been on the possibility of crystallisation from protein solution with high purity. A very limited cases of selective crystallisation were described. Ghatak and Ghatak reported selective crystallisation from protein mixture realised by specific additives (salts) accompanied with charged nano-patterned surfaces.\textsuperscript{31}
study by our group reported that mesoporous nucleants with specific pore size, ordered structure, and narrow pore size distribution are able to promote selective protein crystallisation via controlling the nucleation process.$^{32, 33}$ Still, in all the cases mentioned above the concentration range in this study was at a relatively low level which may not be applicable for fast purification purposes. Judge et al. studied protein crystallisation in the presence of protein impurities,$^{23, 34}$ and, preferential separation of lysozyme from lysozyme – ovalbumin mixtures was achieved by seeded batch crystallisation.$^{35}$ Nonetheless, in the above cases, only a limited range of mixture composition was covered and the only one of the proteins in the solution can be crystallised out under the solution condition. Systematic knowledge of crystallisation behaviour of target protein from the mixture is still absent. Few information is available for introducing seeds/heterogenous nucleants and future scale-up to proceed to selectively crystallise target protein from a more complex solution. The reported preferential crystallisation cases only focused on specific scenarios either with ultralow protein concentration or with a very limited range of protein composition of the mixture.

In this study, we provide the first direct experimental evidence that bio-separation is practical via crystallisation from a binary protein mixture where both proteins are supersaturated and crystallisable under an identical crystallisation condition.

2. Methodology
**Materials.** Lysozyme from chicken egg white (≥ 90%), thaumatin from *Thaumatococcus daniellii*, 1,4-Piperazinediethanesulfonic acid (PIPES) (≥ 99%), potassium sodium tartrate tetrahydrate (99%), sodium chloride ((≥ 99%), and sodium acetate anhydrous ((≥ 99%) were purchased from Sigma-Aldrich (Dorset, UK). Sodium hydroxide (> 98.5%) and hydrochloric acid (37% w/w) was purchased from VWR (Lutterworth, UK). Deionised water was obtained using PURELAB Chorus 1 water purification system (ELGA LabWater). All chemicals were used as received without further purification.

**Protein Crystallisation.** Hanging-drop vapour-diffusion (HDVD) crystallisation method was used in this study. Sodium chloride (NaCl) precipitant solution (5 mg/mL to 100 mg/mL) was prepared by dissolving NaCl in 0.1 M sodium acetate (NaAc) Buffer, pH 4.8. Potassium sodium tartrate tetrahydrate precipitant solution (28 mg/mL to 560 mg/mL) was prepared by dissolving potassium sodium tartrate tetrahydrate in 0.1 M PIPES buffer, pH 6.8. All precipitant solutions were filtered through 0.22 µm Millex-GS Syringe Filter Units (Millipore) before crystallisation trials. Protein (lysozyme/thaumatin) solution was prepared by dissolving the protein powder into the buffer solution which was the same buffer as used for precipitant preparation. Protein concentration in the solution was determined by Nanodrop Onec microvolume UV-Vis spectrophotometer (Thermo Scientific™) at 280 nm using mass extinction coefficient (ε1%) of 26.4 L/g-cm for lysozyme and 12.7 L/gm-cm for thaumatin.36 Protein solution with expected concentration higher than 100 mg/mL was diluted before measured by UV-Vis spectrophotometer. Lysozyme-
thaumatin mixtures were prepared by mixing lysozyme solution and thaumatin solution with determined concentrations. All protein solutions were filtered through 0.22 µm syringe filter (VWR) before crystallisation trials.

HDVD crystallisation experiments were conducted in 24-well VDX™ plate with sealant (Hampton Research). Each well was filled with 500 µL precipitant solution as reservoir solution. A 4 µL droplet with equal volume of protein solution and precipitant solution (same as the reservoir solution) was deposited on a borosilicate cover glass (VWR). The cover glass with the protein-precipitant drop on was carefully inversed and sealed onto the well filled with reservoir solution. The crystallisation plates were then placed into the incubator (20 °C ± 0.5 °C). The plates were observed using CX41 optical microscope (Olympus) regularly after they were set-up. Microscopic images were captured using a GXCAM HICHROME-MET camera (GT Vision).

In this study, we determined the crystallisation results based on the observations of the droplets under the optical microscope. And the droplets were categorised into (1) no crystal, (2) precipitation, (3) only lysozyme crystal(s), (4) only thaumatin crystal(s) and (5) both lysozyme crystal(s) and thaumatin crystal(s). Due to the limitation of the maximum amplification of the optical microscope, only crystals larger than about 5 µm can be observed and the shape of the crystal can be recognised, i.e. result was marked as ‘(5) both types of crystals’ providing at least one lysozyme crystal larger than 5 µm and at least one thaumatin crystal larger than 5 µm were observed in the droplet at the same
time. Considering the inherently poor reproducibility of protein crystallisation and the enormous crystallisation conditions tested, it was not feasible to exam every single crystal using characterisation techniques such as single crystal X-ray diffraction which can be low-throughput and excessively time-consuming to provide representative analysis for the whole sample population. Usage of microscopic images of the droplets is a fast and robust way to screen and track the crystallisation conditions in limited time and the results are real-time, relatively consistent and representative since all the droplets were examined rather than single crystals were sampled and tested off-line. To enhance the confidence level of our results, each condition was repeated at least 12 times to mitigate the inherently poor reproducibility of protein crystallisation due to the stochastic nucleation event. In the first run, experiments were repeated 12 times in 3 separate plates. If the same results were obtained from all the replicates, the condition was only repeated for 12 times. In the second run, another 12 replicates of each condition were repeated. If 22 replicates were given the same results, providing about 95% confidence level, the condition would be only repeated 24 times. For the remaining conditions, more replicates would be tested up to 144 times. In general, conditions with protein concentration lower than 50 mg/mL were repeated in 48 to 144 droplets. Considering the higher degree of supersaturation of the proteins would reduce the fluctuation and uncertainties of the crystallisation results, conditions with higher protein concentration were repeated at least 12 to 24 times to reassure the accuracy of the results.
3. Results and Discussion

**Determination of Protein Crystallisation Condition in HDVD Crystallisation Experiments.** The results in Table 1 show that lysozyme and thaumatin were able to be crystallised individually from their single-protein solutions against crystallisation condition in which potassium sodium tartrate tetrahydrate was used as precipitant. Yet, under the conditions investigated in this study, no thaumatin crystal was obtained by using sodium chloride as precipitant. The droplets remained clear or only precipitations were observed in the period of observation.

**Figure 1** shows illustrative images of the crystallisation droplets crystallised using potassium sodium tartrate tetrahydrate as precipitant. The shapes of thaumatin crystals and lysozyme crystals grown using this precipitant were different. In the range of concentrations investigated in this study, tetragonal lysozyme crystals (**Figure 1A**) were obtained from lysozyme solution while bypiramidal thaumatin crystals (**Figure 1B**) were obtained from thaumatin solution. These two types of protein crystals can be distinguished under the optical microscope by their crystal shapes. **Figure 1C** was captured after Izit Crystal Dye (Hampton Research, US) was added to the droplet. The dye test allows a quick test to see whether the crystals obtained were protein crystals or salt crystals. Protein crystals can be stained via dye molecule diffusion into their solvent channels since protein crystals are less packed compared to small molecule crystals like salts in the buffer solutions. In **Figure 1C**, both types of crystals are stained blue and thus
they were protein crystals. And the bipyramidal shape thaumatin crystals possesses a deeper blue colour while the tetragonal lysozyme crystals possesses a lighter blue colour which may due to the different crystal mosaicity. **Figure 1C** reveals that both lysozyme crystals and thaumatin crystals can be crystallised out from a lysozyme-thaumatin mixture using the tartrate salt as the precipitant while still possessing distinct crystal shapes. Thaumatin crystals remained as bipyramidal shape in the mixture. Lysozyme crystals were tetragonal crystals though defects might be detected under certain conditions. Therefore, further experiments where preferential crystallisation from lysozyme-thaumatin binary protein mixture was attempted were conducted by using potassium sodium tartrate as precipitant rather than sodium chloride.

**Table 1.** Summary of crystallisation experimental results from pure thaumatin solution and pure lysozyme solution under different crystallisation conditions used in this study

| Precipitant solution | Protein Type | Temperatur e | | Protei n Type | Temperatur e | Precipitant | Protein Type | Temperatur e | Precipitant |
|----------------------|--------------|--------------|---------------------|---------------|--------------|---------------------|---------------------|--------------|---------------------|
| Sodium Chloride in 0.1 M Sodium Acetate Buffer, pH 4.8 | Tetragonal Crystal(s) | 4 °C | Lysozyme | 4 °C | Tetragonal Crystal(s) |
| | Tetragonal Crystal(s) | 20 °C | | | |
| Potassium Sodium Tartrate Tetrahydrate in 0.1 M PIPES Buffer, pH 6.8 | Clear/Precipitation | 20 °C | Thaumatin | Clear/Precipitation | Bypiramidal Crystal(s) |
| | Bypiramidal Crystal(s) | 4 °C | | | |
Figure 1. Representative images of lysozyme crystals and thaumatin crystals crystallised against 141 mg/mL potassium sodium tartrate tetrahydrate precipitant solution. A: 50 mg/mL lysozyme. Only tetragonal lysozyme crystals in the droplet; B: 50 mg/mL thaumatin. Only bipyramidal thaumatin crystals in the droplet; C: 50 mg/mL thaumatin + 50 mg/mL lysozyme. Both lysozyme and thaumatin crystals in the droplet and this photo was captured after Izit Crystal Dye (Hampton Research, US) was added to the droplet. All the transparent crystals absorbed dye molecules and turned to be blue afterward. The bipyramidal thaumatin crystals were darker compared to lysozyme crystals.

Protein Crystallisation from Binary-Protein Mixture. HDVD crystallisation method was used in this stage of crystallisation condition screening processes to investigate an operating window for crystallisation from the model mixture. An initial precipitant concentration of 141 mg/mL potassium sodium tartrate tetrahydrate in the buffered solution and temperature at 20 °C were kept the same for the whole set of experiments. The crystallisation plates were observed regularly and for simplicity of the diagram, results listed only included 6 hours, 24 hours, and 48 hours after the plates were set up. The crystallisation period was kept relatively short (days) because of the operation time in the
future scaling-up experiment would not be run for weeks due to the stability of protein solution.

Four crystal occurrence domains were observed from the crystallisation droplets as shown in Figure 2: 1) clear domain with no crystal formation in which no crystal > 5 µm was detected (crystal smaller than 5 µm was not detectable using the optical microscope used in this study), 2 & 3) target domains with only one type of protein crystals (lysozyme crystals or thaumatin crystals only) and 4) mixture domain with a mixture of both types of protein crystals. Figure 2 shows the evolution of crystallisation domains over time. The results also advise that, one type of protein was going to be crystallised out first from the mixture and followed by the other protein later. And the sequence was depending on the mixture composition. This suggests that within a certain period, harvesting a single type of protein crystals from the mixture for separation purpose is possible. Comparing the crystallisation results of single protein solution and results of protein mixture, Figure 2 also reveals that the presence of another protein in the mixture would normally hinder the crystallisation process of the target protein. For instance, after 6 hours, crystals were observed in droplets containing 50 mg/mL lysozyme, but it remained clear after the same amount of time when more than 20 mg/mL of thaumatin existing on top of the lysozyme in the droplet.
**Figure 2.** A schematic illustration of the protein crystal domains of crystallisation from lysozyme-thaumatin mixture in this study. The time denotes the moment from the crystallisation plates were set up. The close symbols with only one colour represent 100% of the droplets in the study had the stated results. The symbols with two colours followed the results with the highest possibility. Circle symbols (grey) represent experimental results where the droplets remained clear with no crystals observed; square symbols (green) represent experimental results where only lysozyme crystals were observed in the droplet; triangle symbols (red) represent results where only thaumatin crystals were observed in the droplet; star symbols (black) represent results where both lysozyme crystals and thaumatin crystals were observed in the same droplet. The shaded regions in the figure only serve as a visual guidance to highlight the domains where different situations were observed.
After 6 hours, a clear droplet domain existed in low protein concentration range with lysozyme concentration $\leq 40$ mg/mL and thaumatin concentration $\leq 30$ mg/mL. In this region, the degree of supersaturation was relatively low and thus crystallisation process was slow. However, after 2 weeks, crystals were observed in this region. Additionally, this clear region reached out to the range of lysozyme concentration of 50 - 60 mg/mL with a thaumatin concentration $\leq 30$ mg/mL. In this extended part, though lysozyme crystals were observed from pure lysozyme solution with the same initial lysozyme concentration, no lysozyme crystallised out as the thaumatin was present in the mixture. Furthermore, when lysozyme concentration was 50 - 60 mg/mL with a thaumatin concentration $> 30$ mg/mL, there was still no lysozyme crystals observed. Lysozyme crystallisation was inhibited within 6 hours due to thaumatin in the mixture. A similar tendency was observed when the initial concentration of thaumatin was 40 – 60 mg/mL, when a higher amount of lysozyme presents in the mixture, no thaumatin crystals were observed after 6 hours while thaumatin crystals were observed in droplets with the same initial thaumatin concentrations. Thaumatin crystallisation was inhibited due to high amount of lysozyme in the solution. With a higher initial lysozyme concentration ($> 70$ mg/mL), lysozyme always crystallised out regardless of the thaumatin concentration in the mixture in the range studied in this work. In the mixture containing both high concentrations of lysozyme and thaumatin, a mixture of both types of protein crystals were observed.
After 24 hours, the mixture domain expanded, and protein crystals were observed in the regions with lower initial protein concentrations. Thaumatin crystal only domain still existed for all mixture droplets with a initial lysozyme concentration no more than 30 mg/mL. However, the domain with only lysozyme crystals almost retreated to the region where no thaumatin was added from the beginning, i.e. pure lysozyme solution.

After 48 hours, the clear region shrank dramatically to the region where the initial lysozyme concentration was no greater than 10 mg/mL. So did the lysozyme crystal only domain and thaumatin crystal only domain. There was no domain with only lysozyme crystals in all the mixture compositions tested. Additionally, the domain with a mixture of both protein crystals expanded further. Apart from experiments 20 mg/mL lysozyme initially and thaumatin concentration higher than 50 mg/mL, in which only thaumatin crystals were observed after 48 hours, all mixture droplets with lysozyme concentration higher than 10 mg/mL had both types of protein crystals.
Figure 3. Representative images of lysozyme crystals and thaumatin crystals obtained from the crystallisation conditions after 24 hours starting with the same thaumatin concentration (20 mg/mL) but different lysozyme concentrations (A: 0 mg/mL; B: 10 mg/mL; C: 30 mg/mL; D: 50 mg/mL; E: 70 mg/mL; F: 90 mg/mL). A, B, and C have only thaumatin crystals. D, E and F have a mixture of lysozyme crystals and thaumatin crystals which were circled out in D and E.

Figure 3 shows a set of representative microscopic photos of the crystallisation droplets 24 hours after the experiments started, both crystal size and crystal number of thaumatin crystals were reduced dramatically as lysozyme concentration increased. Nevertheless, when the initial lysozyme concentration was higher than 80 mg/mL, the number of
thaumatin crystals increased. This increase may due to that lysozyme crystallisation was faster resulting from the high degree of supersaturation. Consequently, free lysozyme in the solution decreased and thus thaumatin crystallisation was less affected by lysozyme in the mixture. Another assumption is based on the nature of protein crystal that protein crystal retains relatively high solvent content comparing to small molecule crystals\(^{37}\). Therefore, as more protein crystals formed from the mixture, less solvent was left in the mixture and thaumatin concentration might have increased accordingly.

Furthermore, as shown in Figure 2, when the initial thaumatin concentration was 10 – 20 mg/mL with a moderate lysozyme concentration of 30 – 70 mg/mL, the reproducibility of HDVD crystallisation results was the worst. The success chance of crystallisation of each type of protein was not 100% and thus lead to an overlap of clear, target, and mixture domains. As shown in Figure 3 (D and E), in this composition region, due to the presence of lysozyme in the solution, both crystal size and crystal number of thaumatin crystals were reduced significantly. Lysozyme crystallisation dominated in this region and there were chances that thaumatin did not crystallise when the initial thaumatin concentration was low.

In general, as shown in Figure 2, there was always one type of protein crystallised out from the solution first and then followed by the other protein crystals. And the sequence was decided by the composition of the mixture, i.e. degrees of supersaturation of the proteins. When enough time was provided, both lysozyme and thaumatin would
crystallise out from the mixture. This suggests that when operation time was controlled properly, bio-separation can be achieved via preferential protein crystallisation even if protein impurity in the mixture was supersaturated and able to be crystallised out under the crystallisation condition.

We also suggest that the presence of another protein, acting as an impurity in the solution, will slow down the crystallisation process of both the target protein and the impurity protein itself. Still, the crystallisation process was not inhibited completely. Additionally, in the model system studied in this work, we did not find evidence that the existing lysozyme protein crystal could stimulate thaumatin crystallisation or vice versa. Hence protein crystals as seeding remains as an option to facilitate target protein crystallisation from the mixture without the risks of promoting the impurity crystallisation simultaneously.

4. Conclusion

In this study, we successfully demonstrated preferential protein crystallisation using lysozyme-thaumatin binary mixture as the model. Four domains were identified: 1) clear domain, 2 & 3) target domains with only one type of protein crystals (lysozyme crystals or thaumatin crystals only) and 4) mixture domain with a mixture of both types of protein crystals. These domains depended on the mixture composition and shifted with time. There was no direct evidence in this study that protein solubility was changed due to the existence of protein impurity. In the model binary protein mixture, the presence of another
protein impurity can slow down the crystallisation process of the target protein. This implies that crystallisation kinetics plays a key role for selective crystallisation from the mixture. Furthermore, as the kinetics of crystallisation can also be manipulated by the presence of the protein impurities, it highlights the importance of further investigation of the effect of protein impurities in more complex crystallisation system. This work demonstrates that protein crystallisation is not only applicable to high-purity protein solution but also a feasible approach to separate a target protein from a more complex mixture environment, even for protein mixtures with both proteins supersaturated. This work also provides a working model system with essential foundation knowledge for future work on protein crystallisation for bioseparation, such as scale-up crystallisation process, seeding, and crystallisation facilitated by heterogeneous nucleants.

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